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GIRTH INCREASE IN WHITE CEDAR STEMS OF IRREGULAR FORM[1]

M. W. BANNAN

Abstract

In coniferous stems the rate of multiplication of fusiform initials in the vascular cambium by means of pseudotransverse division often fluctuates considerably around the circumference. In fluted stems the frequency of these divisions is appreciably higher and the ratio of survival of the newly formed initials relative to the rate of production lower in the depressions than in the adjoining convex portions of the perimeter. The probability that compression is a factor tending to accelerate the frequency of anticlinal division is indicated by the decided increase in rate in areas of the stem subjected to radial pressure. Higher frequencies of pseudotransverse division are accompanied by reduction in mean cell length of the cambial cells and hence of the derived wood elements.

Introduction

In coniferous stems the multiplication of fusiform initials in the vascular cambium, required in the circumferential expansion which accompanies radial growth, takes place by semitransverse division (1, 13). This division, involving the formation of an oblique anticlinal wall near the center of the dividing cell, is usually designated as pseudotransverse, and in the following descriptions will be referred to in abbreviated form as ptr. division. As pointed out in previous publications (2, 28) the frequency of these ptr. divisions is not geared to the rate of growth but is in excess of the theoretical need. The cambium is in a state of flux with new initials coming into being as a consequence of the multiplicative division of the fusiform initials, while others are lost by maturation or reduction to ray initials. The rate of production of new initials and the ratio of survival relative to the frequency of ptr. division fluctuate widely in different parts of the tree. The present paper deals with one of the factors in this variation. All data relate to the eastern white cedar or arbor vitae, *Thuja occidentalis* L.

Cambial Behavior in Grooved Stems

Among the stems utilized for an investigation on the relationship between the frequency of ptr. division in the cambium and the width of the annual rings were some which were out of round, having a shallow depression or groove on one side. In these irregularly shaped stems it was noted that the frequency

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of multiplicative division was actually higher in the areas of circumferential concavity than on either side where the perimeter was convex. The differences in rate of ptr. division in the cambium were reflected in the aspect of the wood as viewed in transection. Thus in Figs. 8 and 9, which illustrate the wood in concave sectors, it will be noted that the cell pattern is in a state of flux, new rows of elements beginning and others ending after short duration. By way of contrast, in the adjoining convex sectors (Fig. 10) the files of elements are of lengthy radial extent and the cell pattern alters only slowly owing to the infrequent ptr. division of the cambial cells. The studies were extended to include stems with deeper grooves, and the trend was confirmed. In 14 of the 16 trees studied the rate of ptr. division was higher in the grooves than on either side (Table I).

TABLE I

FREQUENCY OF PSEUDOTRANSVERSE DIVISION OF CAMBIAL INITIALS PER CM. OF XYLEM INCREMENT AND MEAN CELL LENGTH AT DIVISION IN GROOVES AND ADJOINING CONVEX SECTORS IN THE PERIPHERAL GROWTH OF STEMS, 25–40 CM. IN DIAMETER

Tree No.	Depth of groove, cm.	Groove		Convex sectors	
		Freq. of ptr. division	Mean length cells, mm.	Freq. of ptr. division	Mean length cells, mm.
1	—	2.0	3.17	1.2	3.29
2	0.2	3.3	2.66	2.5	2.78
3	0.4	3.5	3.22	3.5	2.75
4	0.5	3.4	2.90	2.3	3.00
5	1.0	4.4	2.05	1.2	2.85
6	1.0	4.2	2.63	2.5	2.79
7	1.1	3.6	2.48	0.6	2.95
8	1.2	11.2	1.52	4.8	2.30
9	1.5	3.4	2.13	3.4	2.03
10	1.7	6.8	1.91	2.7	2.53
11	2.0	2.0	2.92	1.0	3.14
12	2.1	3.3	2.43	2.3	2.60
13	2.1	13.4	1.46	1.3	3.14
14	2.2	6.0	1.89	1.7	2.93
15	2.2	15.4	1.96	2.1	3.00
16	3.3	8.7	1.84	3.6	2.26
Mean		5.9 ± 4.1	2.33 ± 0.56	2.3 ± 1.1	2.77 ± 0.35

The method of calculating the frequency of ptr. division was as follows. From serial tangential sections of the secondary xylem the location of ptr. divisions in random lineal files of elements was mapped. Some of these case histories as determined from a concave sector are illustrated in Fig. 1, *a–f*, and for the adjoining convex sector in Fig. 1, *g–l*. In ser. *a* it will be noted that seven successive multiplicative divisions occurred during the production of slightly more than 5 mm. of xylem. Thus in this particular series, ptr. divisions occurred at the rate of 14 per cm. of radial increment. In ser. *b* four successive ptr. divisions took place in the development of 2.5 mm. of xylem before the series came to an end, and in ser. *c*, seven successive divisions while 5 mm. of xylem was being laid down. In the several series studied in the grooved area of this particular stem the mean rate was 11.2 successive ptr. divisions per cm. of radial increment of xylem.

In the convexities to either side of the groove the rate of multiplicative division was appreciably lower. For instance, in Fig. 1, ser. *g* and *h*, there were only four successive ptr. divisions during the deposition of 8 mm. of

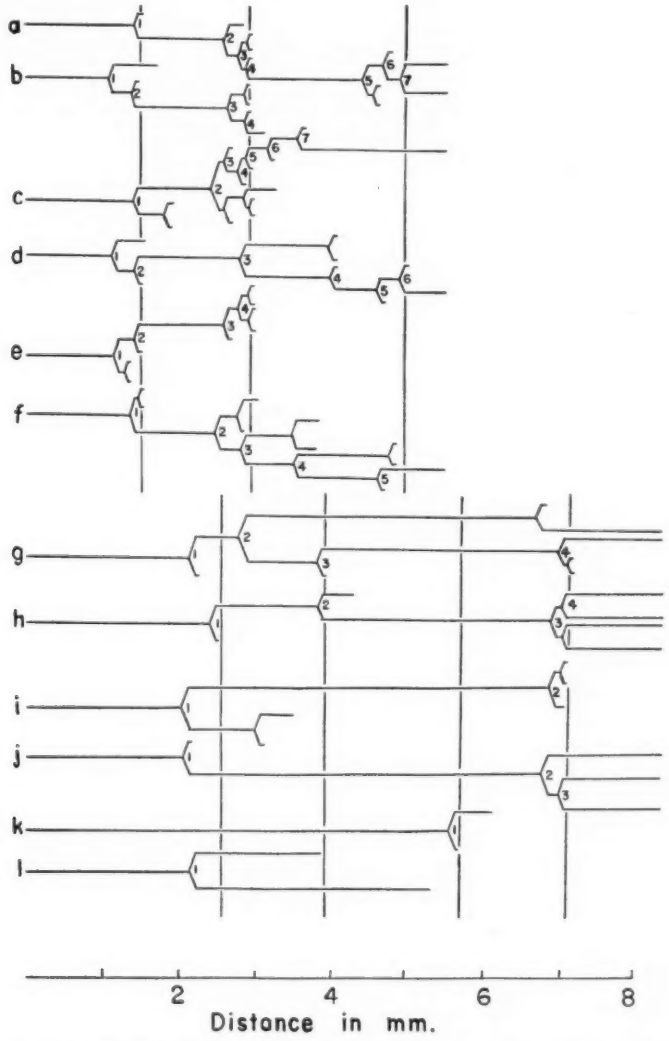


FIG. 1. Diagrammatic representation of the succession of tracheid rows in random lineal series, tree No. 8. The diagrams show the relative time and frequency of pseudo-transverse divisions involved in the production of new fusiform initials in the cambium, and the time of loss of initials. Ser. *a–f* are in a grooved part of the stem and ser. *g–l* in the adjoining convex sector.

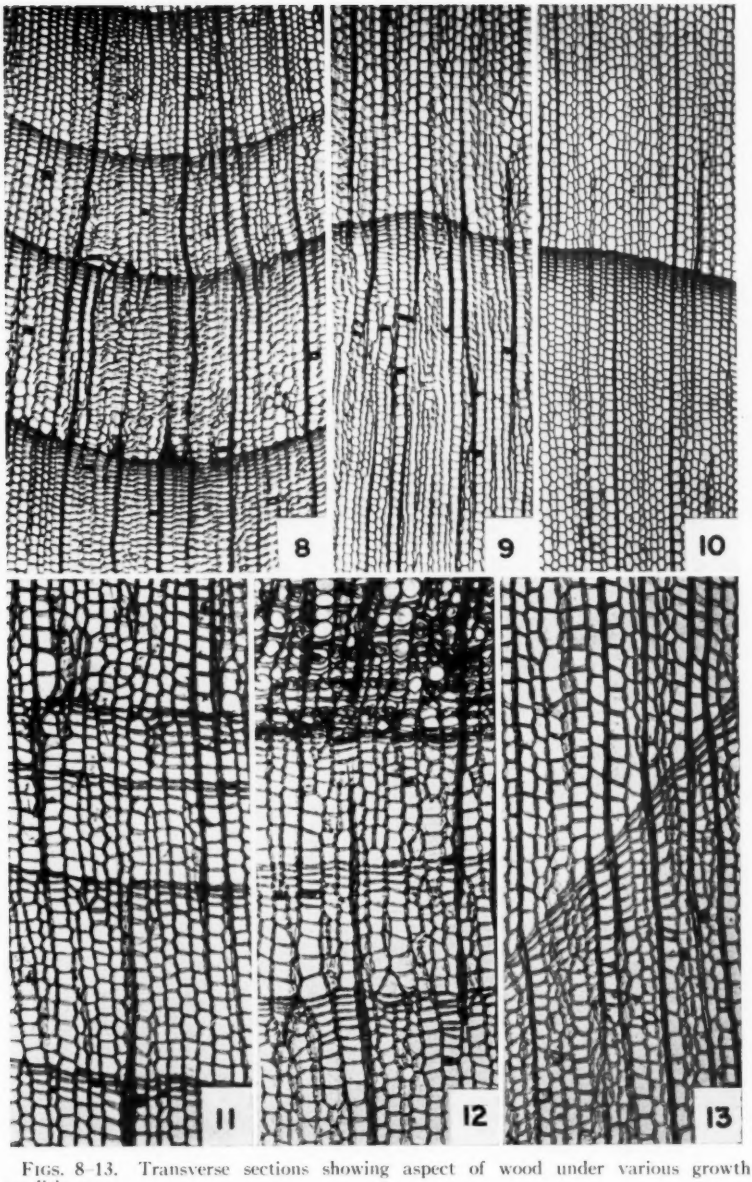
secondary xylem, and in ser. *j* only three. The mean rate in this part of the stem was 4.8 successive ptr. divisions per cm. of radial increment, or less than one-half that in the depression.

As the frequency of ptr. division rose, the mean length of the cambial cells at the time of division decreased. In Fig. 2 it is seen that in the grooved area, where the frequency of ptr. division was 6 per cm., the cells averaged only 1.89 mm. long. In the convex sectors to either side, the mean frequency was much lower at 1.6 and 1.7 per cm. and the cell length at division appreciably greater at 2.85 and 3.01 mm. Fig. 3 illustrates another example. The inverse relationship between cell length and frequency of ptr. division is also recorded in the summaries of Table I.

It is clear that as the frequency of ptr. division rises the divisions tend to occur earlier in the repetitive cycle of cell elongation and division. However, the decrease in cell length is not directly proportional to the elevation in frequency of division. A comparison of the means in Table I shows that a 156% gain in the frequency of division in the grooves as compared to the neighboring convex parts of the perimeter was accompanied by only a 19% drop in cell length. In other words, higher frequencies of ptr. division are accompanied by an increase in the rate of cell elongation between divisions which compensates in part for the higher rate of division. Calculations based on the frequency of anticlinal division and the cell length at the time of division show that the rate of elongation between successive divisions in the depressions was 2.2 times that in the adjoining convexities. Thus it is apparent that in the grooved sectors there is increased stimulation both of ptr. division and of cell elongation.

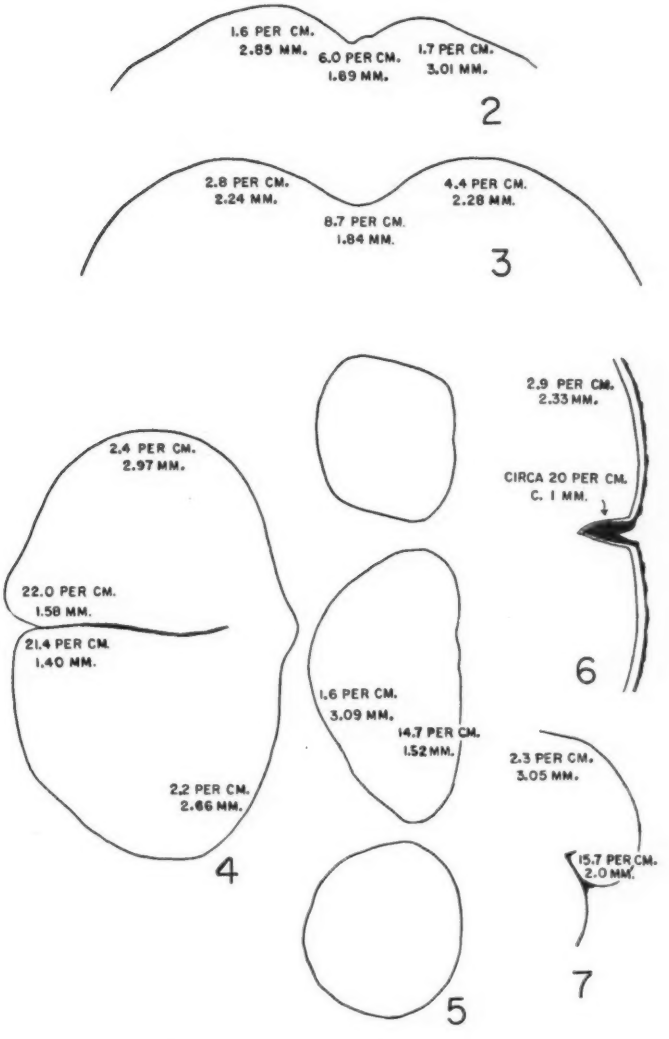
The question arises as to the cause of the stimulation to cell elongation and anticlinal division in the grooves. The aspect of the wood in these sectors varies somewhat. In some cases the rays and radial files of tracheids follow a parallel course, but in other trees there is convergence of the rays and net loss in the number of radial files of elements (Fig. 8). In the latter, the expansion of the convex arcs on either side of the groove would seem to have the effect of placing the cambium in the depression in a state of tangential compression. Sometimes the contour of the rings in the groove is marked by minor folds (Fig. 9). That pressures of various sorts do influence cambial behavior is indicated in some observations which will be set forth below. However, before dealing with these, reference will be made to some other noteworthy features of cambial activity in the grooves.

In Fig. 1, ser. *a–f*, where case histories are diagrammed for six lineal series in a grooved area, it is seen that after some 40 ptr. divisions the net result is a reduction in number of fusiform initials from 6 to 5. Despite the high rate of multiplicative divisions in the cambium the loss of initials by maturation or reduction to ray initials is so great that there is often a net loss in number of cambial cells. This, together with the reduction in mean cell length which is a concomitant of the accelerated ptr. divisions, results in a contraction



FIGS. 8–13. Transverse sections showing aspect of wood under various growth conditions.

FIG. 8. Wood in groove (tree No. 15, Table I). FIG. 9. Same (tree No. 8, Table I). FIG. 10. Wood in adjoining convex sector of the same tree. FIG. 11. Wood on inner compressed side of forked stem. FIG. 12. Xylem and phloem in closed fissure (see Fig. 6). FIG. 13. Wood in groove showing inclination of files of tracheids to ring boundary.



FIGS. 2–7. Diagrams presenting data on the fluctuations in frequency of pseudo-transverse division per cm. of xylem increment and mean cell length at the time of division in stems of different types.

FIG. 2. In depression and adjoining convex sectors in grooved stem (tree No. 14, Table I). FIG. 3. Same for another grooved stem (tree No. 16, Table I). FIG. 4. In the inner compressed and outer free-growing sides of a forked stem. FIG. 5. In the plane and opposite convex sides of a stem flattened against a fence rail. FIGS. 6 and 7. In stems with closed fissures.

of the surface area of the cambium. By way of contrast, in the laterally adjoining convex sectors the ratio of cell survival is much higher so that a gain accrues. This is shown in Fig. 1, ser. *g–l*, where the number of cambial initials increased from 6 to 9.

Another interesting feature of cambial behavior in the concave sectors is the change in cell shape which occurs during the growing season. As noted by Newman (20) the radial files of elements in the depressions of fluted stems often intersect the stem perimeter at an angle (Fig. 13). In such cases the tangential walls of the late wood elements are inclined so as to lie parallel to the circumference, but in the remainder of the growth ring the tangential walls are in a different plane, being oriented to meet the radial walls at a right angle (Fig. 13). It is apparent that on renewal of cambial activity in the spring, and coincidental with the widening of the zone of periclinal cell division, the cross-sectional shape of the cambial cells alters from that of a parallelogram to a rectangle. As growth slackens in the late summer, and the zone of division narrows, reversion occurs to the original parallelogramic form.

Cambial Behavior in Stems Subjected to Radial Pressure

An impressive illustration of the effect of pressure on cambial activity was discovered in a roadside tree deformed by contact of many years' duration with a cedar fence rail. The rail, which was part of an old-style fence reminiscent of pioneer days, was so firmly fixed at either end as to apply pressure against the tree during the greater part of its life span. The trunk, which was $5\frac{1}{4}$ in. in diameter below the point of contact and $4\frac{3}{4}$ in. above, was planoconvex in transection in the contact area with a major diameter of $7\frac{1}{2}$ in. (Fig. 5). Cambial behavior with respect to girth increase differed on opposite sides of the stem at the level of contact with the rail. During the growth of recent years in the side flattened against the rail, ptr. divisions occurred at the very high rate of 14.7 per cm. of xylem increment and mean cell length at division was only 1.52 mm. (Fig. 5). In contrast, on the opposite convex side of the stem, where growth was apparently normal, anticlinal divisions took place at the rate of only 1.6 per cm. of xylem production and mean cell length at division was 3.09 mm.

Another manifestation of the influence of pressure on the cambium was found in a forked stem. On the inner side, where the twin trunks were applying pressure against each other as a consequence of their radial growth, the frequency of ptr. division was much higher and the mean cell length lower than in the outer, free-growing side (Fig. 4). Fig. 11 illustrates the wood structure on the inner, compressed side.

Further examples of the effect of pressure were found in two trees which bore longitudinal fissures caused possibly by lightning. The convergent overgrowths from either side had trapped some of the old bark between and this compacted mass was providing resistance to further growth. As shown by the data in Figs. 6 and 7, ptr. divisions followed in rapid sequence

in the approaching overgrowths. The inconstancy in cell pattern, due to the continuous formation of new initials and the loss of older ones, is illustrated in Fig. 12. Length of the cells was markedly reduced as compared with that in normal growth to the side.

In the four cases described, where radial growth was achieved against resistance, the effect of the increased pressure on the cambium was to stimulate ptr. divisions to several times the normal rate. As a consequence of the accelerated rate of ptr. division the mean cell length was markedly reduced. Wardrop (27) noted a reduction in cell length after the application of pressure to two small Douglas fir trees. The effect in that instance was to produce wood with cells having thicker walls, a flatter micellar spiral, and a reduced length. Wardrop concluded that the shorter tracheids arose as a result of increased ptr. divisions in the cambium. This deduction is confirmed in the present observations.

The influence of pressure on stem growth has been studied intermittently during the past 70 years. After applying ligatures de Vries (26) noted a decrease in ring width and an increase in number of thick-walled, fibrous elements. Krabbe (16) also observed a diminution in ring width after the application of pressure, but in his experimental material the proportion of late wood, and hence of thick-walled cells, was reduced. A characteristic feature of conifers is the development under certain circumstances of wood with thick-walled, rounded cells. This so-called red wood, compression wood, or reaction wood has been attributed by some investigators to compression (9, 24), but others have disagreed and have suggested a variety of alternative factors as its cause. Reviews of the extensive literature on the subject have been provided by Kienholz (12) and Spurr and Hyvärinen (25). Among later workers Hartmann (10) and Sinnott (23) concluded that reaction wood is not formed as a direct response to compression or gravity but is regulatory in character.

In the material of *Thuja occidentalis* examined by the author the tracheids in the areas of compression were notably shorter than in other sectors, a feature of similarity with reaction wood, but the xylem was not otherwise altered and did not resemble reaction wood in appearance. The cells were characteristically rectangular in cross section, the cell walls were no thicker and in late wood were possibly slightly thinner than in ordinary wood, and the late wood did not exceed the usual proportion of the annual ring. As illustrated in Figs. 11 and 12, the most noteworthy features of the wood were a reduction in ring width and a much abbreviated duration of most of the radial files of elements owing to the high frequency of ptr. division.

While the xylem cells did not exhibit obvious alterations in structure in the areas of compression, the phloem was notably different. As is well known, the secondary phloem of *Thuja occidentalis* is characterized by a regular alternation of elements in which every second cell is usually parenchymatous and every fourth cell is a fiber. Moreover there is generally a continued reduction in wall thickness in the successive tangential bands of fibers, from

very thick in the spring to relatively thin in the late summer. In the regions of compression, however, only sporadic thick-walled fibers occurred (Fig. 12). Nearly all possessed only slightly thickened walls with reduced lignin content. This is in agreement with observations of Newcombe (19), who found a delay in the formation of hard bast in *Vicia* and *Urtica* stems grown in plaster casts. While less conclusive, experiments of Pennington (21) and Bücher (7) revealed a reduction in wall thickness in herbaceous stems subjected to compression.

Radius of Curvature and Frequency of Anticlinal Division

In the mass of data accumulated during the extensive investigations on ptr. divisions in the cambium of conifers there is no indication that the frequency of anticlinal division is directly geared to circumferential expansion. Fluctuations occur which are not evidently related to the rate of diameter increase. For instance in Table I it is seen that the frequency of ptr. division varied from 0.6 to 4.8 per cm. of xylem increment in the convex portions of the perimeter of the trees studied, all of which had a diameter in the range of 25–40 cm. Often there was local stimulation of growth so as to produce peripheral convexities with radius of curvature substantially less than that of the stem as a whole. Sometimes the arc of curvature in one sector was notably different from that in another, but the fluctuations in frequency of anticlinal division did not follow the differences in radius of curvature. For instance, in the final growth of the stems referred to, all with diameter of 25–40 cm., the frequency of ptr. division was 2.3 ± 1.3 per cm. of xylem addition in sectors where the radius of curvature was only 3–5 cm., and 2.2 ± 1.2 where the radius was 5.5–13 cm.

The data indicate that a local acceleration of growth, with consequent reduction in radius of curvature, is not accompanied by a rise in frequency of ptr. division. The rate of division always seems to be in excess of actual need so that an ample production of new fusiform initials occurs under all circumstances. There are, however, marked differences in the ratio of survival. In arcs of rapid growth the proportion of newly formed cambial cells that survives is usually higher than in other sectors. In grooves, on the other hand, the survival ratio is very low.

The effects of a local acceleration of radial growth on the rate of ptr. division seem to be felt, not within the arcs of expansion, but rather in the adjoining depressions. In the various specimens where the grooves were less than 2 cm. deep the mean frequency of ptr. division was 4.9 per cm. of xylem increment, as compared with a frequency of 8.1 where the grooves exceeded 2 cm. in depth. No doubt compressive forces were stronger in the deeper, narrower depressions.

Discussion

Because of its importance to the lumber industry the problem of stress in timber has received much attention. Koehler (15) noted the tendency for the outer part of the stem to expand tangentially, as demonstrated by

the closure of radial saw cuts. He interpreted this phenomenon as indicating "that the outer part of a green tree trunk is in compression tangentially". Jacobs (11) and Boyd (5) have also commented on the existence of circumferential compression in the outermost layers of wood. In a review paper Clarke (8) concluded that stress and strain are normal concomitants of growth in trees. Both Clarke and Koehler suggested that the magnitude of the stress may vary with growth conditions.

The emergence of irregular growth patterns and the establishment of local centers of increased cambial activity in the enlargement of stems apparently set in motion forces which further influence growth behavior. From the evidence presented in this article it would seem that compression, whether tangential or radial, has the effect of increasing the frequency of anticlinal division of fusiform initials in the vascular cambium. Not only is the interval between successive pseudotransverse divisions shortened, but the rate of cell elongation between divisions is accelerated. In other words, in sectors subjected to increased pressure there is stimulation of both anticlinal division and cell elongation. Periclinal cell division, on the other hand, tends to be retarded.

Changes in the frequency of semitransverse division of fusiform initials are reflected in alterations in length of the cambial cells and their xylem and phloem derivatives. As the frequency of pseudotransverse division rises the mean cell length drops, but the relationship is not a strictly inverse one. The reduction in cell size in sectors of accelerated anticlinal division is less than might be expected because of an increase in rate of elongation of the cambial initials between successive pseudotransverse divisions. Another counteracting factor is the continued selection of the longest cells in the intracambial competition for survival (4). This is a particularly important factor in areas of frequent semitransverse division owing to the low ratio of cell survival.

As mentioned earlier, it is believed (5, 8, 11) that the peripheral wood of a growing stem is in a state of circumferential compression. While the actual degree of compression may vary at different levels through the cambium and the developing xylem, it is to be expected that all layers are experiencing some compression. The frequency of pseudotransverse division in the cambium is apparently influenced by the magnitude of the compressive forces in that area, but the question arises as to why this reactivity is a property of the cambial initials but not of the derived tissue mother cells which are also a part of the lateral meristem. Only a small percentage of pseudotransverse divisions in the cambial area take place outside the initiating layer, even though the zone of periclinal division is sometimes several cells wide (3, 6, 14, 17, 18, 22, 29).

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STUDIES ON THE MECHANISM OF DEPOSITION AND EXTENSION OF PRIMARY CELL WALLS[1]

G. SETTERFIELD AND S. T. BAYLEY

Abstract

Walls of elongating parenchyma and epidermal cells of *Avena* coleoptiles and onion roots were studied by autoradiography, using C_{14}-labelled sugars, and by electron microscopy of thin transverse sections. The autoradiographs showed that deposition of cellulose takes place over the whole length of the cell, ruling out the possibility of bipolar tip growth. Autoradiographs of cells grown for short times in labelled sugar gave no evidence of localized incorporation of isotope around primary pit-fields, but rather indicated that deposition of microfibrils is finely dispersed over the entire wall surface. The thin sections revealed a marked change in wall structure as elongation proceeds. Walls of young cells contain only transversely oriented microfibrils while older walls include an inner region of predominately transversely oriented microfibrils bounded outside by a region with microfibrils showing an irregular transition to longitudinal orientation. The degree of longitudinal orientation in the outer region increases with cell length. These results for thin walls are consistent with the multi-net model for wall growth deduced by other workers from electron microscope studies on cell fragments. Thin sections have also revealed layering in several types of growing walls which is difficult to interpret on the basis of the multi-net model alone.

Introduction

Several theories have been proposed to explain the mechanism of cell wall, and more particularly cellulose, deposition during cell elongation. One notable difference between these theories lies in the localization of the regions where cell wall synthesis is assumed to occur. The theory of bipolar tip growth proposed by Mühlethaler (13) for parenchyma of *Avena* coleoptile, and later generalized by Frey-Wyssling (7, 8), restricts new wall formation to cell tips. On the other hand, Wardrop (24) and Scott *et al.* (21), studying parenchyma of coleoptiles and onion roots respectively, suggested that addition of microfibrils takes place at pit-fields and plasmodesmata. Two other theories have been proposed which assume that synthesis of wall material takes place over the entire cell surface. In the mosaic theory, which is based on studies of elongating coleoptile parenchyma, Frey-Wyssling and Stecher (9, 23) suggest that microfibrils are added by intussusception at points where the wall is transiently penetrated by cytoplasm. The multi-net theory, on the other hand, supposes that microfibrils are added predominately by apposition to the inner surface of the wall. This theory was originally advanced by Roelofsen and Houwink (11, 20) to account for the structure of walls in certain seed hairs and the pith cells of *Juncus*. It has recently been applied to *Avena* coleoptile and onion root parenchyma by Wardrop (25, 26).

Autoradiography, using labelled sugars, offers a technique for determining where new wall material is incorporated and thus for making at least a partial

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evaluation of the validity of these divergent theories. Recently, Wardrop (25, 26) and Gorham and Colvin (10) used this approach in a study of cell elongation in *Avena* coleoptiles. The present report describes the result of similar, more extensive studies, begun independently, on both *Avena* coleoptiles and onion roots. In addition, thin sections, which allow a new approach to the study of fine structure of walls by electron microscopy (2, 14), were used in the present study to interpret and extend results obtained from autoradiographs.

Methods and Materials

Autoradiography

Segments of *Avena sativa* coleoptiles (Lanark variety) were prepared and grown by the methods given in detail by Nitsch and Nitsch (15). Segments 4 mm. long were cut 3 mm. below the tips of 15 to 20 mm. long coleoptiles. The segments were floated on 1 p.p.m. manganese sulphate for 3 hours and then transferred onto 1 ml. of medium containing 0.01 *M* potassium phosphate – citrate buffer (pH 5.0), 8 p.p.m. indoleacetic acid, 2% unlabelled sucrose, and sufficient, generally C_{14}-labelled, sucrose (specific activity 5.04 μc. per μM.) to give a total radioactivity of 25 μc. per ml. The small amount of labelled sucrose did not significantly affect the rate of growth of the segments as compared with that of segments grown in only 2% sucrose. The segments were incubated in this medium in the dark at 23° C. on a slow rotary shaker. Segments were removed at various times from 15 minutes to 22 hours after the start of culturing, measured as to length, and killed in 80% ethyl alcohol.

For onion root studies, small pickling bulbs were placed over water until a good crop of roots 15 to 20 mm. in length had germinated. Very short and long roots were removed and the lengths of the remaining roots measured. The bulbs were then placed over vials so as to immerse the roots in water containing a mixture of C_{14}-labelled glucose, fructose, and sucrose, of unknown relative concentration (specific activity 2.91 μc. per μM. as glucose), with a total radioactivity of 25 μc. per ml. The roots were allowed to grow in this solution in light at 23° C. The rate of growth was the same as that of control roots grown in water alone. Roots were removed at various times from 15 minutes to 24 hours after immersion in the isotope solution, measured as to length, and killed in 80% ethyl alcohol.

After they were killed, the coleoptile segments were cut into pieces 1 to 2 mm. long which were passed through subsequent treatments without regard to position in the original segment. With the onion roots the terminal centimeter was cut into ten 1 mm. pieces which were carried through subsequent operations separately.

The tissue pieces from both coleoptiles and roots were passed through the following extraction procedure.

1. 80% ethyl alcohol, 12–24 hours, 23° C.
2. 80% ethyl alcohol wash, 10 minutes.

3. Absolute ethyl alcohol: conc. hydrochloric acid 1 : 1, 24 hours, 5° C.
4. Three washes in water, 10–15 minutes each.
5. 0.5% ammonium oxalate, 24 hours, 5° C.
6. Three washes in water, 10–15 minutes each.
7. 17.5% potassium hydroxide, 24 hours, 23° C.
8. Three or more washes in water, 10–15 minutes each.

This extraction was designed to remove non-cellulosic materials from the cell without disturbing the cellulose component. After extraction, cell walls appeared devoid of protoplast in the phase-contrast microscope. As a further check on the procedure, segments of onion roots grown in water were extracted, frozen, fragmented (21), and examined in an electron microscope. Although some of the fragments contained residual amorphous material (Fig. 1) the majority consisted only of relatively clean cellulose microfibrils (Fig. 2). A micro-Kjeldahl analysis of extracted coleoptile segments showed the presence of approximately 0.15% nitrogen on a dry weight basis (10). It was concluded that most of the wall material remaining was cellulose, although some background radiation might have been contributed by small amounts of contaminating, non-cellulosic polysaccharides. The possibility of incorporation of radioactivity through exchange into cellulose synthesized prior to treatment with labelled sugar is ruled out by the studies of Ordin *et al.* (17).

In preparation for autoradiographs, the extracted tissue pieces were teased apart on "subbed" slides and squashed in water beneath a cover slip. The cover slip was removed using dry ice (5) and the slide with adhering tissue was washed in 95% ethyl alcohol for 5 minutes and then air-dried. Autoradiographs were prepared by the stripping film technique; both A.R. 10 plates (Kodak Ltd., London) and 35 mm. Permeable Base Stripping Film (Eastman Kodak, Rochester) were used and found satisfactory. The autoradiographs were exposed for 26 days at 40° F., developed in Kodak D-19 (6 minutes for the English film and 2 minutes for the Eastman), fixed, and dried. They were mounted in water for study by bright-field and phase-contrast microscopy.

Electron Microscopy

Thin sections for electron microscopy were prepared using the methods described in detail by Bayley *et al.* (2). Segments about 1 mm. in length were cut from the mid-region of coleoptiles from 3 to 40 mm. long (germinated on damp filter paper in the dark) and from 1 to 6 mm. from the tip of 20 mm. long onion roots (germinated in water). The segments were treated with 10% pectinase solution (4) at pH 4 for 6–12 hours, fixed in 1% osmium tetroxide buffered at pH 7.4 (18), dehydrated, and embedded in methacrylate. Transverse sections (silver to gold by reflected light) were mounted on grids, the embedding medium removed with amyl alcohol and acetate, and the sections shadowed at 15° with palladium–gold alloy.

Results

Autoradiography

The amount of growth of coleoptile segments in the solution containing labelled sucrose is shown in Table I. During the total culture period of 22 hours the average length of the segments increased from 4 mm. to 9.5 mm. indicating that the cells increased their length by approximately 138%.

Representative autoradiographs obtained for both parenchyma and epidermal cells in the coleoptiles are shown in Figs. 3 to 5. After growth for 1 hour in labelled sucrose, the walls of both parenchyma and epidermal cells produced a weak autoradiograph with the silver grains randomly scattered along the entire length of the cell (Fig. 3). There was no sign of localized uptake either at cell tips or at pit-fields. At later times the autoradiographs increased in density but the grains remained evenly dispersed (e.g. Fig. 4). After 12 hours it was possible to detect a slightly heavier incorporation of isotope in the thick outer walls of the epidermal cells (Fig. 5). Occasionally, clumps of epidermal cells showing no incorporation were encountered; these presumably represented cells that were killed or wounded in the preparation of the original coleoptile segments.

The course of growth of the onion roots in the solution of labelled sugars is shown in Table I. The quantitative aspects of cell elongation in roots are considerably more complicated than in coleoptiles because cells in different regions of the root undergo different relative amounts of elongation (22). The roots do, however, have the advantage of possessing cells in all stages of elongation. A further complication arises with roots since cellular differentiation occurs during elongation. The majority of observations in the upper regions of the root were made on cortical parenchyma and epidermal cells.

After 30 minutes treatment with labelled sugars, cells in the lower regions of the elongation zone showed a very weak autoradiograph with the silver grains randomly distributed (Fig. 6). As time of treatment with labelled

TABLE I

GROWTH OF *Avena* COLEOPTILE SEGMENTS AND ONION ROOTS IN SOLUTIONS CONTAINING RADIOACTIVE SUGARS

Time in labelled sugar solution	Mean increase in length, mm.	
	Avena coleoptile segments, original length 4 mm.	Onion roots
15 min.	0.5	—
30 "	1.0	—
1 hr.	1.0	0.4
2 "	1.3	0.5
3 "	1.5	1.3
6 "	2.3	2.0
9 "	—	1.9
12 "	3.0	2.4
22 "	5.5	—
24 "	—	4.0

sugar increased, cells further up the root showed this light incorporation of isotope also. Photographs of cells with pits were traced and the tracings matched with photographs of their weak autoradiograph. No evidence for association of silver grains with pits, or groups of pits, was found. After 3 hours' growth in labelled sugar, cells from all regions of the root showed a relatively strong autoradiograph (Figs. 7 and 8) of uniform density and this situation continued to later times with corresponding increase in autoradiograph intensity. The heavy incorporation of C_{14} by cells near the end of their elongation (8–10 mm. from the root tip) suggests that reinforcement of the wall was occurring.

Two types of cell, viz. elongated root-cap cells (Fig. 9) and epidermal cells from the region of cell maturation, showed little incorporation of labelled sugars at the later times. The behavior of these cells, along with that of the dead, non-radioactive epidermal cells found in the coleoptile preparations, indicated that adsorbed isotope did not contribute significantly to the autoradiographs.

Electron Microscopy

Pectinase treatment has a twofold advantage in studies on cell wall structure. It causes the cytoplasm to collapse away from the wall (in vacuolated cells) and removes much of the non-cellulosic wall material, thereby revealing the microfibrillar structure. In sections, the arrangement and orientation of microfibrils becomes particularly evident when the wall section "topples over" during removal of embedding medium. The relative lengths of the microfibril segments in such sections give a direct indication of their orientation *in situ* (2). Orientations described below refer to the major axis of the cell.

Fig. 10 is a low magnification picture of a typical transverse section of a coleoptile parenchyma cell. Figs. 11 to 14 are higher magnification pictures of transverse sections of portions of parenchyma walls from coleoptiles of various lengths. In the 3 mm. coleoptile (Fig. 11) the fibrils all appeared to be essentially transversely oriented (long segments). By the time the coleoptile had reached a length of 10 mm. however, a change had occurred and the walls showed a tightly packed layer of microfibrils oriented transversely on the inside with a loose, less well oriented mat of fibrils on the outside (Fig. 12). At the very outside of the wall some very short microfibril segments, indicating a longitudinal orientation, were seen. In the sections of parenchyma of the 40 mm. coleoptile (Fig. 13) the outer disoriented region contained even more very short microfibril segments, indicating that the frequency of microfibrils approaching longitudinal orientation increased as elongation proceeded. Distinct layers of microfibrils were found in the inner tangential wall of the epidermal cells and in walls of the subepidermal cells of 10-mm. coleoptiles (Fig. 14). The profuse layering of the outer wall of the epidermal cells has already been described (2).

Sections of parenchyma cell walls in the onion root showed a similar type of structural change as elongation proceeded. About one millimeter from the tip (Fig. 15) the microfibril arrangement was like that in the 3-mm.-long coleoptile: loosely woven, predominantly transversely oriented. In sections cut from the third and fifth millimeters from the tip (Figs. 16 and 17) the picture was similar to that in the longer coleoptiles, with a well-defined inner region of predominantly transversely oriented microfibrils and an outer, looser mass of microfibrils varying from transverse to longitudinal orientation. In the fifth millimeter from the root tip, distinct layers of microfibrils were found in many parenchyma cells (Fig. 17).

The thickened outer walls of the epidermal cells 1 millimeter from the root tip showed an extreme form of the type of structure found in elongated parenchyma cells; they had several distinct layers of transverse microfibrils bounded on the outside by several layers of longitudinal microfibrils (Fig. 18). Epidermal cells farther up the root were heavily encrusted with amorphous material and revealed no internal structure even after pectinase treatment.

Discussion

Before the autoradiographic data is discussed, it should be pointed out that the autoradiographs indicate only the position and relative amount of new material deposited in the wall. Deposition need not be accompanied by surface extension, but may serve only to increase the thickness of the wall. Thus, it is possible for the extension of the wall to vary in different regions of the cell although an autoradiograph with an even distribution of silver grains is obtained.

The most striking feature of the autoradiographs was their indication, in all cells studied, of an even deposition of cellulose over the entire surface of the wall. This result does eliminate the possibility of bipolar tip growth as interpreted by Frey-Wyssling (7), where new wall is deposited only at cell tips by the advancing protoplast. Wardrop (25, 26) and Gorham and Colvin (10) came to a similar conclusion from autoradiographic studies on coleoptile cells. The possibility that surface extension occurs only at the cell tips despite the even deposition of new wall material, as discussed above, is ruled out by studies on pit distribution in coleoptile and onion root parenchyma, by Wardrop (24) and Scott *et al.* (21), and by growth measurements on epidermal cells of coleoptiles by Castle (3). Wilson (27) also concluded from studies on the distribution of wall perforations in the parenchyma of *Elodea canadensis* stem that tip growth does not occur. Although differential elongation at cell tips may occur in certain types of cells such as root hairs (16) or cambial initials (1), it is probably not a general mechanism in primary growth of plant tissues.

A second important line of evidence comes from the autoradiographs of cells grown for short times in labelled sugar where the position of the first detectable incorporation of isotope could be observed. These autoradiographs showed a light, random distribution of isotope with no obvious localization at

specific points on the wall surface such as pit-fields. This result suggests that deposition of microfibrils did not take place at relatively large, microscopically resolvable, centers of synthesis as intimated by Wardrop (24) and Scott *et al.* (21). Wardrop (25, 26) in his later, autoradiographic, studies arrived at a similar conclusion but admitted that his results were equivocal; his autoradiographs were obtained only from cells grown for 12 hours in labelled sugar. On the basis of the fact that the walls of parenchyma cells in *Elodea* stem which abut on intercellular spaces contain no pit-fields, Wilson (27) has also concluded that pit-fields are not essential centers of wall synthesis.

Elimination of theories involving well-defined centers of deposition leaves several possible mechanisms for wall addition which cannot be distinguished by autoradiography, since the limit of resolution of this method is about five microns. Addition of microfibrils may take place at well-dispersed, submicroscopic "islands of synthesis" (19), by transient intussusception as in the mosaic theory (9, 23), or by apposition as postulated in the multi-net model (11, 20).

Wardrop (25, 26) recently carried out an electron microscope study of fragmented *Avena* coleoptile and onion root parenchyma. He found that microfibrils on the inner surface of the wall are predominantly transversely oriented whereas those on the outer surface are disoriented and form a netlike structure. Wardrop considers these results consistent with the multi-net theory (11, 20), which postulates that transversely oriented microfibrils are continually added to the inner surface of the wall, while microfibrils on the outer surface are disoriented by the increase in wall surface during elongation.

The electron microscope studies on thin sections presented here show that during elongation there is a definite change in structure of thin parenchyma walls which is consistent with the multi-net concept. In both coleoptiles and roots, the walls of young parenchyma cells contain only transverse microfibrils while the walls of older cells have a region of predominantly transversely oriented microfibrils on the inside and show an irregular transition to longitudinal orientation toward the outside. Furthermore, the frequency of longitudinal microfibrils in the outer, disoriented region increases with increased cell length. These results, taken with those of Wardrop, would suggest that at present the multi-net model best explains the mechanism of growth of thin walls.

Concerning the concepts of small "islands of synthesis" (19) and mosaic growth (9, 23), in numerous observations on thin sections of cells fixed without prior pectinase treatment, no evidence of localized points of microfibril synthesis or of penetration of the wall by cytoplasm (except at pit-fields) has been found. Mühlethaler (14) in an electron microscope study on thin sections of growing walls presented no evidence of "islands of synthesis" and only found dispersed cytoplasmic penetration of the wall in very young cells.

Although the multi-net model satisfactorily explains growth of thin walls, several complications should be mentioned. One of these is the possibility of differential elongation within the same cell, as postulated by Sinnott and

Bloch (22) to explain changing intercellular relationships in the elongation zone of grass roots. As noted above, the autoradiographs for onion roots presented here gave no evidence of localized intracellular incorporation of isotope. This observation has also been confirmed in preliminary autoradiographs of cells of the elongation zone of *Lepidium sativum* roots. Such lack of uneven incorporation of isotope indicates that if differential intracellular enlargement occurs it is not brought about by localized synthesis of cellulose, but rather must be due to localized surface extension accompanied by even deposition of wall material over the whole surface as discussed above. This in turn would suggest that localized intracellular elongation would result from differential plasticity of the wall. On the basis of the multi-net model it would be expected that a greater absolute number of microfibrils, and an increased frequency of transversely oriented microfibrils, would be found in areas of the cell undergoing little elongation as compared with areas showing great enlargement.

Other complications in generalizing the multi-net model arise in connection with the complex wall structure found in certain elongating cells. Examples of such complex wall structure are the layers in the walls of epidermal and subepidermal cells of coleoptiles (2) (Fig. 14), young cortical cells of onion roots (Fig. 17), and collenchyma cells (12, 14). The ribs of longitudinal fibrils in coleoptile parenchyma (13, 24, and 26) are a similar case. These extraneous layers are often classified as secondary wall although all appear to be present during cell elongation. This classification is somewhat misleading since the term secondary wall is usually restricted to wall material laid down inside the primary wall after the cessation of elongation (6). The term "thickened primary" proposed by Majumdar and Preston (12) for collenchyma walls would seem to be useful in describing all the above cases. At any rate, it is not yet clear how the multi-net model alone can account for growth of these layered walls. For example, as pointed out by Bayley *et al.* (2), it is difficult to explain how the regularity of layering in the outer wall of the epidermal cell of the *Avena* coleoptile is maintained if only the multi-net mechanism is operative. However, the slightly increased incorporation of isotope in outer epidermal walls shown in the autoradiographs (Fig. 5) is not nearly that expected if reinforcement of all wall layers with new microfibrils is occurring. The thickened outer epidermal walls of the onion root showed a structure perfectly consistent with the multi-net theory (Fig. 18) and it would be surprising, although not inconceivable, if different mechanisms operated in the two types of epidermal cell. However, further study is necessary before definite conclusions can be drawn.

Acknowledgments

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EXPLANATION OF FIGURES

FIGS. 1, 2. Electron micrographs of fragments of cell walls from onion root extracted by the procedure used in autoradiography. Scale lines represent 1 μ. FIG. 1 shows the amorphous material found in a few instances; in most fragments, this was absent, FIG. 2. FIG. 1, ×26,000. FIG. 2, ×15,000.

FIGS. 3–9. Photomicrographs showing (A) cells under phase contrast illumination and (B) corresponding autoradiographs under bright field illumination. Scale lines represent 20 μ.

FIG. 3. Parenchyma (P) and epidermal (E) cells of a coleoptile segment grown in radioactive sucrose for 1 hour. ×200.

FIG. 4. As Fig. 3, grown in radioactive sucrose for 2 hours. ×330.

FIG. 5. Part of an epidermal cell from a coleoptile segment grown in radioactive sucrose for 12 hours. Note the denser autoradiograph from the thick outer wall (O). ×790.

FIG. 6. Parenchyma cell from the subterminal second millimeter of an onion root grown in radioactive sugars for 30 minutes. ×910.

FIG. 7. Parenchyma cell from the subterminal fourth millimeter of an onion root grown in radioactive sugars for 3 hours. ×470.

FIG. 8. Large parenchyma cell from the subterminal ninth millimeter of an onion root grown in radioactive sugars for 3 hours. ×390.

FIG. 9. Parenchyma (unlabelled arrows) and elongated rootcap (R) cells from subterminal second millimeter of an onion root grown in radioactive sugars for 3 hours. Note the lack of an autoradiograph from the rootcap cell. ×570.

FIGS. 10–18. Electron micrographs of thin transverse sections of cell walls after pectinase treatment; embedding removed, shadowed. Unlabelled arrows point towards the centers of the cells. Scale lines represent 1 μ.

FIG. 10. Parenchyma cell in a 40 mm. long *Avena* coleoptile. Pectinase treatment has caused the cytoplasm (C) to collapse away from the wall (W) into the center of the lumen. ×2000.

FIG. 11. Adjacent walls of two parenchyma cells in a 3-mm.-long coleoptile. The amorphous material is the remains of the cytoplasm. ×17,000.

FIG. 12. Wall of a parenchyma cell in a 10-mm.-long coleoptile. ×16,000.

FIG. 13. Wall of a parenchyma cell in a 40-mm.-long coleoptile. ×34,000.

FIG. 14. The junction between the inner tangential wall of an epidermal cell (E) and the radial walls of two subepidermal cells (S) in a 10-mm.-long coleoptile. Note the layering. ×11,000.

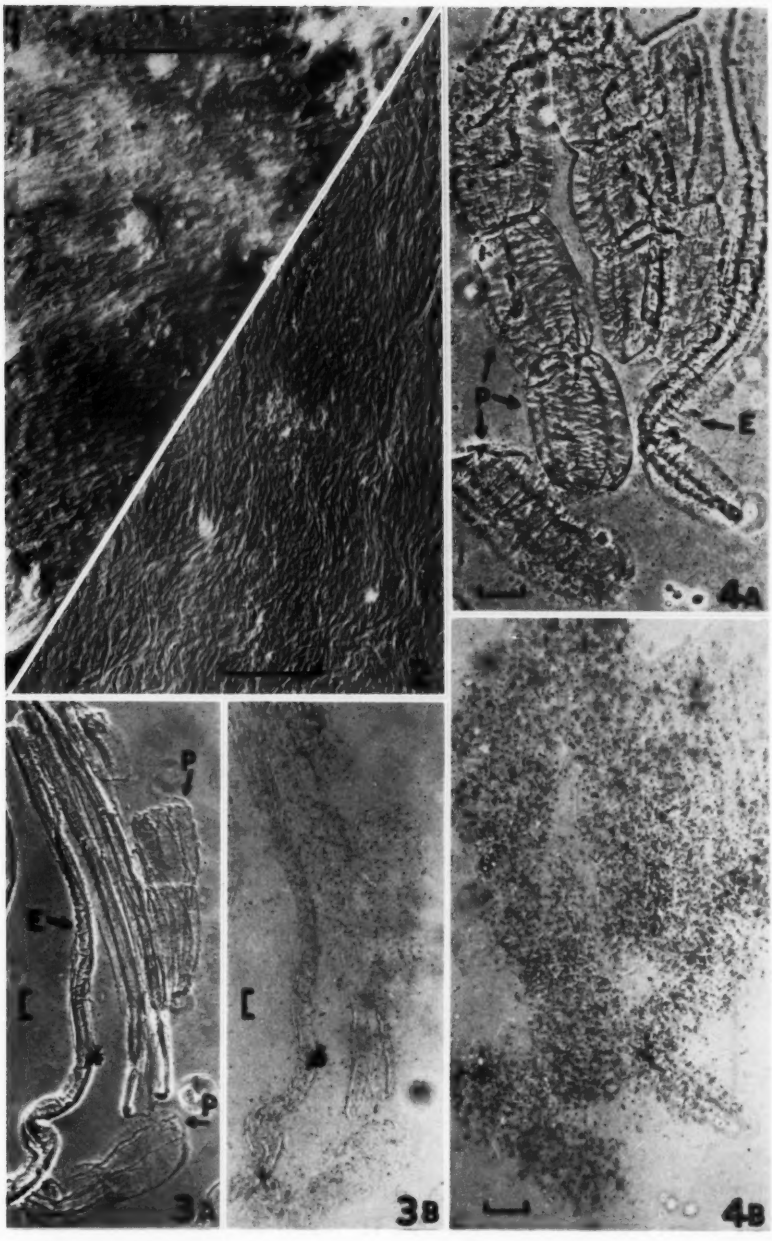
FIG. 15. Adjacent walls of two parenchyma cells about 1 mm. from the tip of an onion root. ×20,000.

FIG. 16. Wall of a parenchyma cell in the subterminal third millimeter of an onion root. ×17,000.

FIG. 17. Adjacent walls of two parenchyma cells in the subterminal fifth millimeter of an onion root. Note the layering (L_1 and L_2). ×20,000.

FIG. 18. Outer wall of an epidermal cell about 1 mm. from the tip of an onion root. ×20,000.

NOTE: Figs. 1–18 follow.



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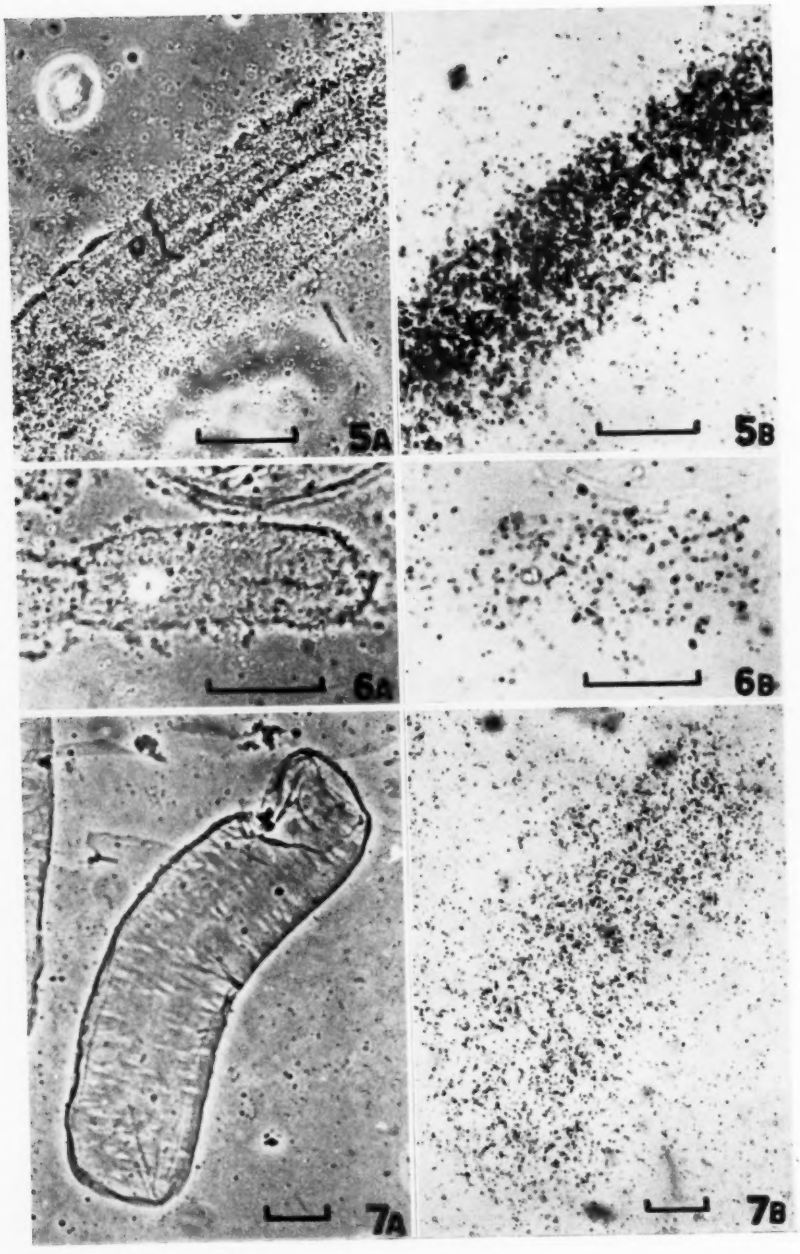

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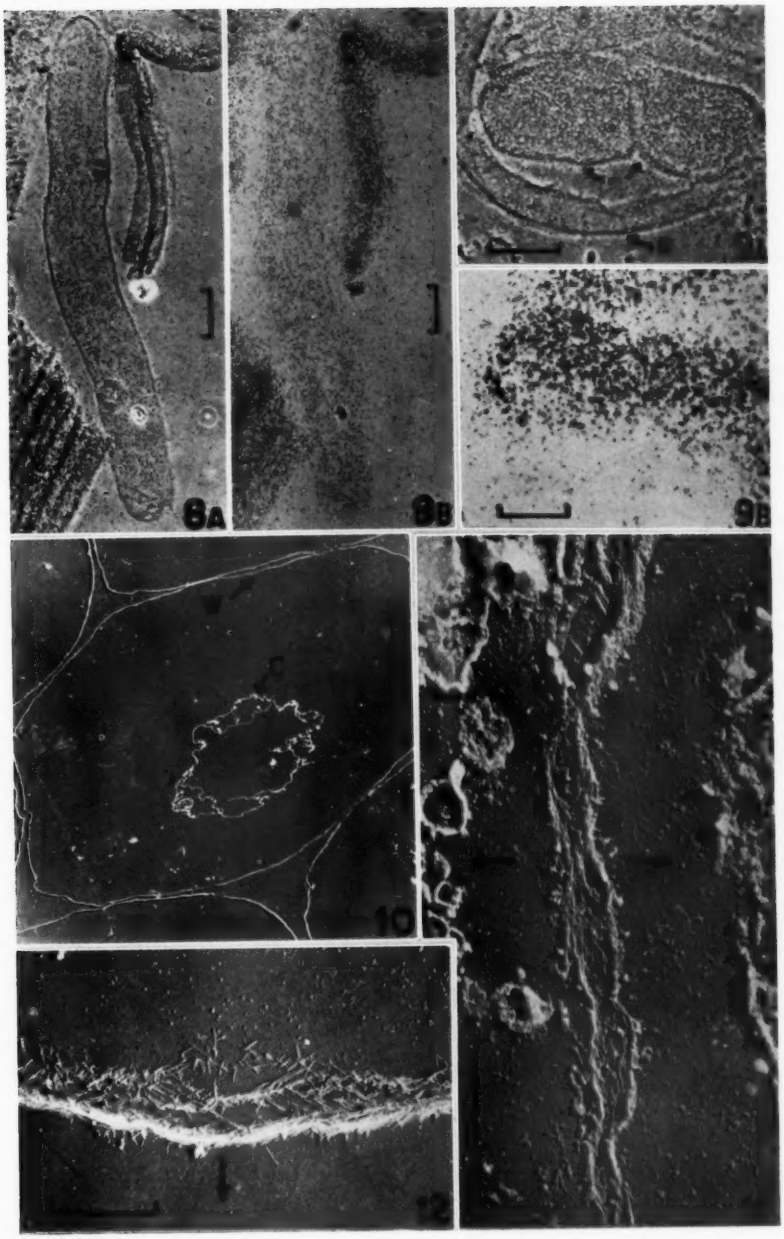

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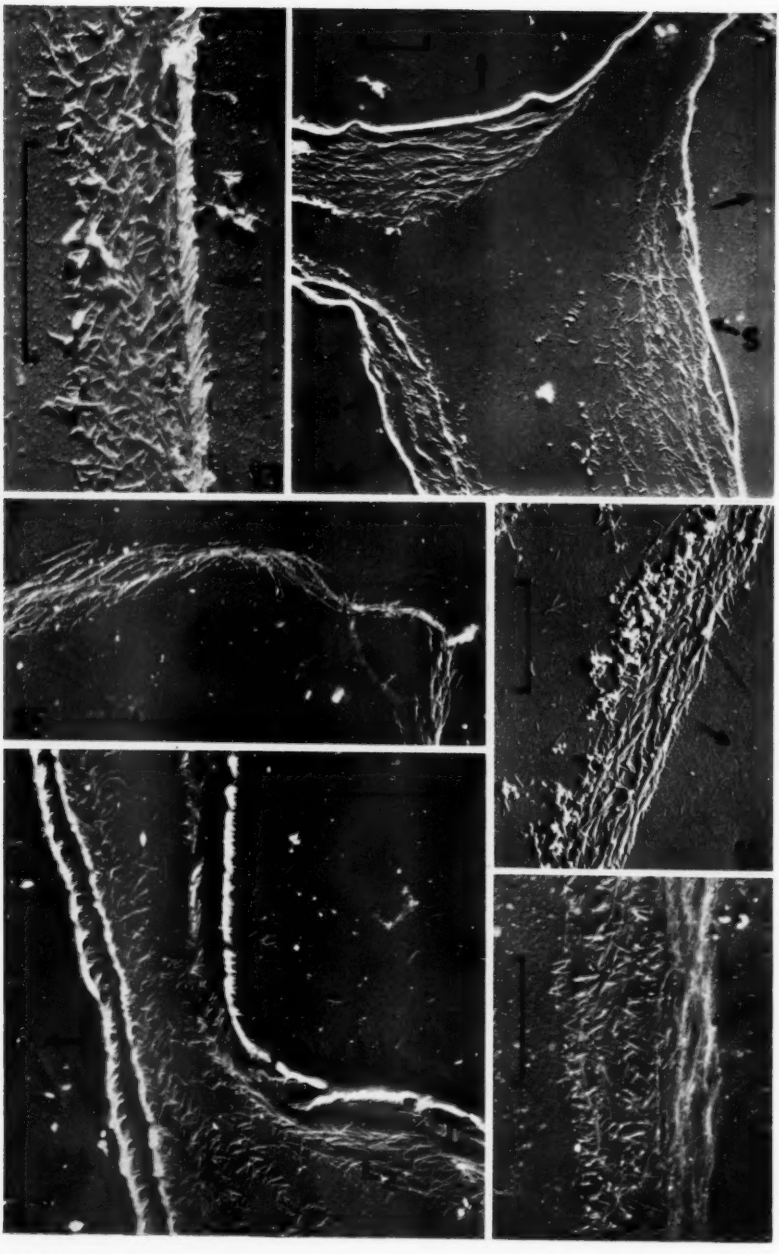

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THE UTILIZATION OF SINGLE ORGANIC NITROGEN COMPOUNDS BY WHEAT SEEDLINGS AND BY PHYTOPHTHORA PARASITICA[1]

WILLIAM NEWTON[2]

Abstract

The growth of wheat seedlings under aseptic conditions is shown to be well supported by L-alanine, glycine, and urea, but not so well as by nitrate. On the other hand, D-alanine and the optical isomers of leucine exerted toxic effects; seedling growth in these solutions was less than in the cultures receiving no nitrogen, and the presence of nitrate in the nutrients did not reduce their toxic effects. However, the toxic effect of D-alanine was partially offset by L-alanine. A somewhat parallel influence upon the growth of wheat seedlings and *P. parasitica* was exerted by D- and L-alanine, D- and L-leucine, and glycine, but not by urea. The latter poorly supported the fungus although it helped the growth of wheat. D-leucine is more toxic to *P. parasitica* than L-leucine, less toxic to wheat seedlings as measured by growth inhibition, but more toxic as measured by chlorotic lesions in the foliage.

Introduction

Little is known about the direct utilization of organic nitrogen compounds by wheat or other higher plants, although the application to soils of materials rich in organic nitrogen is the oldest of the fertilizer practices. The assumption that higher plants secure all their nitrogen from the soil in the form of ammonium and nitrate salts should not be accepted in view of the abundant evidence that urea is utilized by many when applied as a solution to the foliage. It seems probable that in the degradation of organic matter by microorganisms in the soil the release of amino acids and other organic nitrogen compounds would allow their direct absorption by higher plants. This study was prompted by the discovery that the optical isomers of alanine exerted a somewhat parallel effect upon the growth of wheat seedlings and of the fungus *Phytophthora parasitica*. The growth of both was well supported by L-alanine and the dextro form failed to support either (5,6).

Experimental Procedure

The wheat seedlings were grown under aseptic conditions as follows. The seed, var. Thatcher, was soaked for 6 hours in a saturated solution of Arasan and then transferred, without being washed, to oatmeal agar plates to germinate and develop. When the sprouts had reached approximately 5 cm. in length the seedlings were transferred to large sterile test tubes (8 in. × 1 in.) containing 20 ml. of the nutrient solutions, one seedling per tube. The nutrient solutions varied little in composition or concentration except with respect to nitrogen. Hoagland's solution was adopted as the nitrate nutrient and this was modified by the omission of nitrogen in the

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control or 'no nitrogen' series and by the replacement of the nitrate with specific organic compounds in the others. Nitrogen concentrations were made equivalent throughout. The seedlings were grown for 14 days at 60° F. under artificial light.

The *Phytophthora* cultures were grown at 25° C. for 20 days in flasks containing 50 ml. of complete nutrient solution previously described (4), modified only with respect to the organic nitrogen source and adjusted to contain equivalent nitrogen concentrations.

Results

The results are summarized in Table I.

TABLE I

THE INFLUENCE OF NITROGEN COMPOUNDS UPON THE GROWTH OF WHEAT SEEDLINGS AND *Phytophthora parasitica*

Nitrogen compound	Wheat seedlings		*P. parasitica*
	Height (cm.)	Green wt. (mg.)	Dry mycelial mats (mg.)
Control (no nitrogen)	19	83	Nil
Nitrate	29	185	32
DL-Alanine	16	102	99
L-Alanine	28	163	120
D-Alanine	5	62	3
D-Alanine and nitrate	6	60	
L-Leucine	13	42	12
D-Leucine	18	74	4
L-Leucine and nitrate	12	43	
Glycine	24	180	118
Urea	24	163	28
LSD 1% level	4	11	5

Discussion of Results

The data show that the growth of wheat seedlings in the L-alanine, glycine, and urea cultures is greater than in the controls, the cultures dependent solely upon the nitrogen in the seed. The growth, however, is somewhat less than in those supported by nitrate. On the other hand, the growth in the cultures containing D-alanine or either of the optical isomers of leucine is markedly less than in the controls. Consequently, these effects may be defined as toxic. It is apparent that these amino acids block the normal metabolism of the wheat plant when they are present in nutrients as single organic nitrogen compounds. Their toxic effects are not diminished by the presence of nitrate.

It is of interest to note, however, that although D-alanine markedly inhibits the growth of wheat seedlings both alone and in the presence of nitrate, D-alanine does not block the utilization of its optical isomer, L-alanine. The growth of wheat seedlings supported by DL-alanine is much greater than that by D-alanine although significantly less than by L-alanine.

It may be noted that there is a parallelism between the influence of organic nitrogen compounds upon the growth of wheat seedlings and upon *Phytophthora parasitica*. The growth of both wheat and the fungus is favored by L-alanine and blocked by D-alanine, and both are inhibited by the isomers of leucine and favored by glycine. This is according to expectations. Plant pathologists are well aware that substances that are toxic to fungi are usually toxic to the tissues of their hosts. Exceptions to this rule are of special interest to pathologists because it may be assumed that in varietal resistance the balance between susceptibility and resistance in some cases may be very delicate and consequently an organic nitrogen nutrient that is favored by the host and not by the fungus may throw the balance in the direction of resistance especially when the nutrient is applied as a spray against leaf fungi. In this study, urea only falls into the exceptional class. This substance as absorbed through the roots of wheat seedlings well supported their growth although not as effectively as nitrate. On the other hand, urea is not utilized readily by *P. parasitica*. It is of interest to speculate, therefore, whether the control of black shank of tobacco by urea drench, as recently reported by Lautz (3), was due to the unfavorable influence of the absorbed urea upon the growth of *P. parasitica*.

The toxic effects of D- and L-leucine upon wheat seedlings and upon *P. parasitica* are of interest for it would appear from the data in Table I that D-leucine is less toxic to wheat than L-leucine and that the converse is true with respect to the fungus. Since the growth of most microorganisms is favored by the laevo forms of amino acids (2), the apparent lower toxicity to wheat of the dextro form may require an explanation. L-Leucine induced

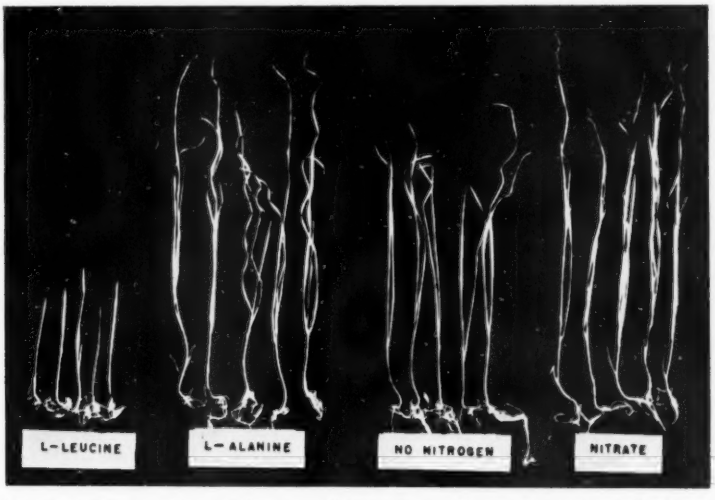

FIG. 1. The growth of wheat seedlings, illustrating support by L-alanine and toxicity by L-leucine as compared with the nitrate and "no nitrogen" controls.

stunting but few abnormal characteristics in the foliage. On the other hand, the D-leucine caused a marked chlorosis of the foliage in the form of streaks although the inhibition of growth was less pronounced. The stunting effect of L-leucine is shown by Fig. 1.

It has already been noted that glycine favors the growth of both the seedlings and the fungus. Apparently higher plants are not all favorably supported by glycine. Audus and Quastil (1) have reported that it is toxic towards cress.

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THE PHYSIOLOGY OF HOST–PARASITE RELATIONS

IV. THE EFFECT OF MALEIC HYDRAZIDE AND INDOLEACETIC ACID ON THE RUST RESISTANCE OF KHAPLI AND LITTLE CLUB WHEATS[1]

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Abstract

Little Club (rust reaction type 4) and Khapli (rust reaction type 1 with race 15B) wheats were grown in sand culture in 1-qt. crocks or 5-in. pots. Daily applications of maleic hydrazide or indoleacetic acid were started 5 days before (10 days after sowing), on the day of, and 5 days after inoculation with stem rust (Race 15B) and were continued for about three weeks. Maleic hydrazide inhibited growth and, at 5 and 10 mg. per pot, flowering. Type 4 pustules developed on Khapli plants treated with 5 and 10 mg. of maleic hydrazide daily from the 5th day before and from the day of inoculation. Indoleacetic acid (0.5 mg. per pot from the 10th day after sowing) stimulated growth and flowering and slightly increased the resistance of Khapli. Treatments started 5 days after inoculation had no effect on rust development on Khapli, and none of the treatments altered the rust reaction of Little Club. Spores produced on plants treated with maleic hydrazide gave normal rust reactions on untreated plants.

The effect of maleic hydrazide on the plants and on rust development was not altered by the simultaneous application of uracil, which reversed the growth inhibition caused by thiouracil. Thiouracil inhibited rust development in the presence of maleic hydrazide.

Introduction

Parts I and II (24, 20) dealt with the accumulation of radioactive substances and the time course of respiration at infections of rust and mildew on resistant and susceptible cereal hosts. In Part III (25) it was further shown that the increase in respiration (OR) was not accompanied by a proportionate rise in fermentation (NR) but was correlated with an increased participation of the pentose phosphate pathway in the respiration of the host tissue. There are many indications that infection with obligate parasites disturbs the normal auxin relations of their hosts. Accordingly, the present paper and Part V (23) are concerned with certain aspects of the auxin relations of cereal leaves infected with rust and mildew. Specifically, this paper describes the effect of indoleacetic acid and maleic hydrazide on the reactions of Khapli and Little Club wheats to Race 15B of stem rust.

Livingston reported that the diethanolamine salt of maleic hydrazide, in concentrations which inhibited the growth of Cheyenne winter wheat, increased the size of pustules of leaf rust (16). Johnson (9) found that DDT markedly increased the susceptibility of Khapli wheat to stem rust (Race 17), a result which was confirmed by Hotson for Race 56 (7). Maleic hydrazide is widely regarded as an antiauxin (15) and inhibits cell division and growth (2, 5, 6). In addition, it produces marked metabolic disturbances which result in the accumulation of sucrose, starch, and free amino acids (4, 18). Similar changes occur in the leaves of Khapli wheat treated with maleic hydrazide (21) and DDT (3).

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Methods

The plants were grown in Ottawa quartz sand in 5-in. pots or 1-qt. crocks in the greenhouse. Each morning the containers were thoroughly flushed with tap water and 100 ml. of Hoaglands No. 2 nutrient solution plus 5 p.p.m. of iron (as Sequestrene: Alrose Chemical Co.) were added to the sand every second afternoon. Approximately 10 days after planting, when the first leaves were fully emerged, the plants were thinned to five per pot and the experimental treatments started. Growth regulating substances were dissolved in water or Hoagland's solution and added directly to the sand without wetting the plants. Indoleacetic acid, uracil, and thiouracil were obtained from Nutritional Biochemicals Corp. Maleic hydrazide (practical grade) was obtained from Eastman Organic Chemicals.

Experimental

Experiment I was carried out with Little Club wheat, which normally gives a type 4 rust reaction. The experimental design is shown in Table I together with the effects of maleic hydrazide and indoleacetic acid on the percentage dry weights of first leaves.

None of the treatments had any visible effect on the development of stem rust (Race 15B), type 4 pustules being produced on all inoculated plants. On the other hand, both maleic hydrazide (all concentrations) and indoleacetic acid (0.5 mg. per pot per day) caused characteristic morphological changes in the treated plants. Maleic hydrazide markedly inhibited growth (see Fig. 1) and strikingly increased the percentage dry matter of the leaves (Table I). The leaves on plants receiving 5 and 10 mg. of maleic hydrazide daily were darker green and thicker than those on untreated plants. Anthocyanins accumulated in the later stages of treatment, the leaves turning red and finally yellow in color. No inflorescences developed and dissection of plants treated with 10 mg. of maleic hydrazide daily from the 5th day before

TABLE I

THE EFFECTS OF MALEIC HYDRAZIDE AND INDOLEACETIC ACID ON THE PERCENTAGE DRY MATTER IN THE FIRST LEAVES OF LITTLE CLUB

	Effect of daily treatment on % dry wt.					
	Maleic hydrazide (mg.)			IAA (mg.)		
Treatment started	10	5	1	0.5	0.1	0.05
Five days before inoculation	17.3	14.9	9.7	9.0	9.0	9.3
Day of inoculation	12.5	10.6	8.8	8.7	8.9	9.2
Five days after inoculation	10.0	9.4	9.2	9.2	8.9	9.1

NOTE: The amounts of maleic hydrazide and indoleacetic acid shown are added daily, in solution, to each pot. Two pots were used for each treatment. Six untreated pots served as controls. Date of planting: 18/11/54. Inoculation of one pot per treatment and three control pots: 3/12/54. Sampling of uninoculated pots: 13/12/54, i.e., 25 days after planting. Per cent dry weight of untreated plants = 9.3.

FIG. 1. The effects of maleic hydrazide (10 mg. per pot per day) and indoleacetic acid (0.5 mg. per pot per day) on the growth of Khapli wheat. Note inhibition of growth by maleic hydrazide (left) and the erect habit of plants treated with indoleacetic acid (right), as compared with untreated plants (center).

FIG. 2. The effect of thiouracil on the growth of Khapli wheat. From left to right, treatments 7, 8, 9, 10 (Table III) and untreated plants. Note the severe inhibition of growth by thiouracil (10 mg. per pot) and its partial reversal by uracil at 10, 25, and 50 mg. per pot.

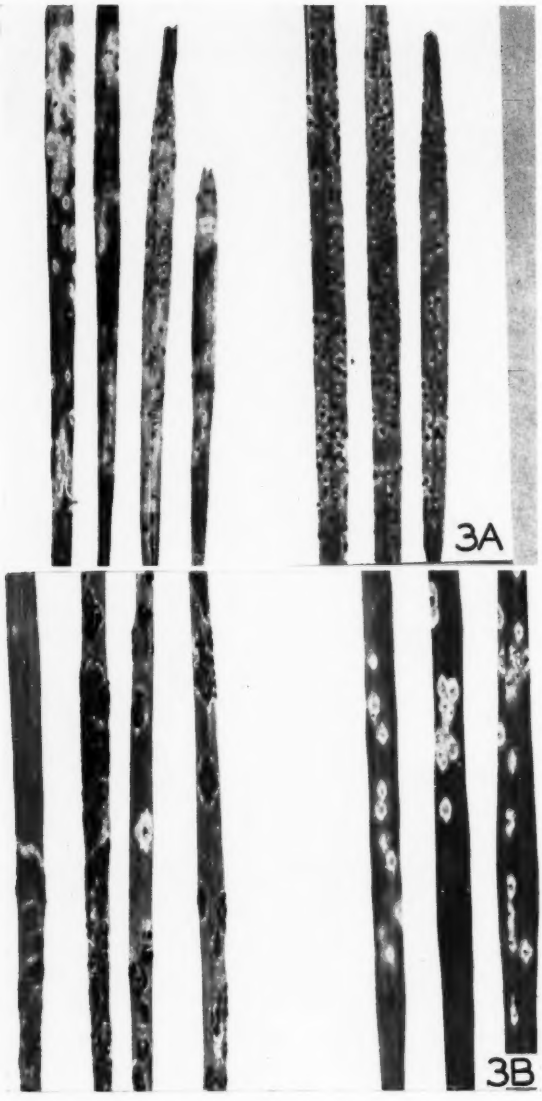

FIG. 3. The effect of maleic hydrazide in breaking the resistance of Khapli wheat to Race 15B of stem rust. Fig. 3A, 10 days after inoculation. Untreated leaves on *left*; treated leaves on *right*. Fig. 3B, 15 days after inoculation. Untreated leaves on *right*; treated leaves on *left*. Treatment (10 mg. MH per pot) started 5 days before inoculation. In Fig. 3B note on third leaf from left one resistant (type 1) infection.

inoculation showed that the apical meristems had disappeared. Presumably those primordia present when the treatment started developed into leaves and no new primordia formed. The number and length of the primary and secondary roots were reduced. No secondary roots developed on those plants receiving 10 mg. of maleic hydrazide daily from the 5th day before inoculation. The root tips were bulbous, rather than tapered as in untreated plants.

Plants treated with indoleacetic acid (0.5 mg. per day) from the 5th day before and from the day of inoculation were a light green in color. The leaves were more erect and narrower than those on untreated plants (see Fig. 1). The number and length of primary and secondary roots was increased. There were no appreciable effects on the percentage dry weight of the leaves (Table I).

Experiment II, identical in design with Experiment I, was carried out with Khapli wheat, on which light infections of stem rust (see 20) normally produce type 1 pustules. Ten days after inoculation the pustules on plants treated with 5 and 10 mg. of maleic hydrazide daily from the 5th day before inoculation were scored as type 3^{--} and 3^{-}, respectively. Four days later the formation of secondary uredinia was observed at many of the infections on the first and second leaves of these plants and also of those treated with 10 mg. of maleic hydrazide daily from the day of inoculation (see Fig. 3). Sporulation at these infections was luxuriant. Spores from these infections gave normal (type 1) rust reactions on untreated plants. Treatments with maleic hydrazide started 5 days after inoculation had no effect on rust development.

The infections on plants treated with 0.5 mg. of indoleacetic acid daily from the 5th day before inoculation were highly necrotic and there was almost no sporulation, although the plants were only lightly infected (see 20). The reaction type was scored as 1^{-} to 1^{--}. On all other plants not treated with maleic hydrazide a type 1 reaction was obtained.

The general morphological effects of the treatments were the same as those already described for Little Club and are shown in Fig. 1. The data in Table II illustrate the effect of maleic hydrazide in increasing the fresh and dry weight of the first leaves. About 75% of the increase in fresh weight is accounted for

TABLE II

THE EFFECT OF MALEIC HYDRAZIDE ON THE FRESH AND DRY WEIGHTS OF THE FIRST LEAF OF KHAPLI

Treatment	Dry wt.* (mg.)	Fresh wt.* (mg.)	% dry wt.
A. Nil	10.25	119.8	8.56
B. Maleic hydrazide (5 mg. daily)	27.40	139.4	19.66
C. Maleic hydrazide (10 mg. daily)	27.75	146.1	18.99
D. Mean of B + C	27.58	142.75	19.32
E. D − A	17.33	22.95	10.76

*Per 100 leaf discs (diameter 2.8 mm.). Samples taken after 15 days of treatment.

by the increase in dry weight per unit area. In this experiment the treatments were continued until the untreated plants produced inflorescences. Those plants receiving the higher concentrations of maleic hydrazide did not flower. On the other hand, indoleacetic acid stimulated flowering. Flowering occurred earlier and the spikes were larger than in the untreated plants (see (8)). Quantitative data were not obtained in this experiment, but were obtained in a subsequent experiment which will be described separately (26).

It will thus be clear that indoleacetic acid stimulated the growth and development of Khapli and possibly increased its resistance to rust. Maleic hydrazide inhibited growth, prevented or retarded flowering, and dramatically reduced resistance.

Experiment III was carried out with Khapli to determine whether the effect of maleic hydrazide was due to an interference with the metabolism of uracil. Uracil and maleic hydrazide are structural isomers (10). The experimental treatments and results are summarized in Table III. The data show, once again, the effect of maleic hydrazide in reducing the rust resistance of Khapli. The effect of maleic hydrazide was not reversed by uracil (Treatments 3, 4, and 5, Table III). Thiouracil retarded the development of both the plants and the fungus. Its effects were partially reversed by uracil (Treatments 7–10, Table III, Fig. 2). Moreover, thiouracil very much reduced the effect of maleic hydrazide in breaking resistance (Treatment 11, Table III). These results suggest that maleic hydrazide does not interfere with the metabolism of uracil.

TABLE III

THE EFFECTS OF MALEIC HYDRAZIDE, URACIL, AND THIOURACIL ON THE GROWTH AND RUST REACTION OF KHAPLI WHEAT

Daily treatment	Growth effects on uninoculated plants		Rust reaction
	Days after start of treatment		
	5	20	
1. Nil	—	—	1
2. MH 10 mg.	—	—	4
3. MH 10 mg. + uracil 10 mg.	—	Plants severely stunted.	4
4. MH 10 mg. + uracil 25 mg.	—	First leaves yellowing	4
5. MH 10 mg. + uracil 50 mg.	—	at tips. Anthocyanin formation	4
6. Uracil 10 mg.	—	—	1
7. Thiouracil 10 mg.	Plants stunted. Leaves pale green with chlorotic flecks	Plants severely stunted. Chlorotic mottling. Some albinism	0–1⁻
8. Thiouracil 10 mg. + uracil 10 mg.	Symptoms progressively	Symptoms less marked	0–1⁻
9. Thiouracil 10 mg. + uracil 25 mg.	less marked than in 7.	than in 7. Least in 10.	1⁻
10. Thiouracil 10 mg. + uracil 50 mg.	Least in 10	Strong tillering. Some albinism	1
11. Thiouracil 10 mg. + MH 10 mg.	Plants stunted. Leaves pale green with chlorotic flecks	Plants severely stunted	1+

NOTE: Three pots per treatment. Planted 28/8/55. Treatments started 7/9/55. Inoculated with Race 15B 12/9/55.

Discussion

Alterations in the level of endogenous indoleacetic acid following infection of cereal leaves with rust and mildew may play a role in regulating the mobilization of metabolites at infections of obligate parasites (23), and in the

development of hypertrophy and hyperplasia where these occur as a result of infection. Assuming this, if maleic hydrazide is an antiauxin (15) or reduces the concentration of endogenous indoleacetic acid below some effective level by stimulating the activity of indoleacetic acid oxidase (17), it might be expected to prevent the development of rust on a susceptible host such as Little Club. In fact, maleic hydrazide has no such effect. Indeed, it dramatically decreased the resistance of Khapli wheat to stem rust. It is now important to determine whether varieties which are more resistant than Khapli are similarly affected by maleic hydrazide.

Klein and Klein (11) have shown that maleic hydrazide, in concentrations which depress the growth of tomato plants, has no effect upon the development of tomato crown gall tumors (see also (12)). By comparison with nontumorous tissue, such tumors contain large amounts of indoleacetic acid, which Klein and Vogel (13) have shown to be necessary for the duplication of crown gall tumor cells. Kulescha (14), Pilet (19), and Audus and Thresh (1) have all found that maleic hydrazide, in concentrations inhibitory to growth, does not reduce the level of endogenous indoleacetic acid. Apparently the question must remain open whether maleic hydrazide is an antiauxin (1). Audus and Thresh (1) suggest that it may exert a number of different actions depending upon its concentration and the nature of the tissue. The results of Experiment III, while not unequivocal, suggest that maleic hydrazide is unlikely to act as an antimetabolite of uracil. Its effects on the metabolism of leaves appear to be fairly uniform (21).

The metabolic disturbances produced by maleic hydrazide in the first leaf of Khapli (21) bear a general resemblance to those which occur at the loci of type 4 rust-infections on Little Club. These changes are: (i) an increase in dry weight, (ii) an increase in sugars (sucrose) and the formation of starch, (iii) an increase in soluble amino compounds, and (iv) an increase in respiration. On a dry weight basis, the increase in respiration in leaves treated with maleic hydrazide is, however, only about one-fifth of that which occurs in rust infected tissue (20). Moreover, maleic hydrazide does not, like infection with rust, change the apparent degree of participation of the pentose phosphate pathway in respiration, as reflected in the C_6/C_1 ratio (26).

Whatever its specific mode or modes of action, the metabolic changes produced by maleic hydrazide undoubtedly result from its inhibitory effects on cell division (2, 5, 6) and the consequent elimination or inhibition of growing points. Since photosynthesis continues after treatment with maleic hydrazide, carbohydrates and other substances accumulate in the leaves. It may thus be surmised that the general conditions which are normally created by infection of a susceptible leaf with rust (see above) are here created by the suppression of the growing points to which photosynthates are normally transported and, as a result, resistance to rust is broken. It is of considerable interest that DDT, which increases the dry weight, sugar, and free amino acid content of Khapli leaves, also makes them susceptible to rust (3, 9). Similarly,

when the leaves of Khapli are excised shortly after inoculation and maintained with their cut ends in water in the light, they become appreciably less resistant than attached leaves. This point was first brought to our attention by Prof. D. Forward. Heavily inoculated, attached first leaves of Khapli rapidly decline in nitrogen content and, eventually, in dry weight (20). These losses are prevented by excision. Attempts to alter the resistance of Khapli by excising the apical meristem and all leaves except the first leaf, which was inoculated with rust, were not successful. It was, however, impossible to prevent rapid tillering under these conditions and, also, the root meristems remained intact.

It has recently been shown by Schwinghamer (22) that ionizing radiation, in growth inhibiting doses, breaks the resistance of Khapli, Kenya Farmer, and P.I. 94701 (U.S.D.A. accession No.) wheats to race 15B of stem rust, and of oats to oat stem rust and oat crown rust. The shoot apex was shown to be the major radiosensitive site, but radiation of both roots and crown was necessary for the maximum expression of susceptibility. Irradiation of the leaves alone did not decrease resistance. Ionizing radiations destroy indoleacetic acid (13, 27) and Klein and Vogel (13) have shown that the growth inhibiting effects of radiation on crown gall tumors can be prevented by treatment with indoleacetic acid.

Treatment with indoleacetic acid slightly increased the resistance of Khapli to stem rust. If real, this effect is possibly to be attributed to the stimulation of competing growth centers in the host. Infections on susceptible species clearly compete successfully with other metabolic 'sinks' in the host. Evidence of 'competition' between the individual rust pustules on a single leaf has already been presented (20).

It is clear that the data presented in this paper do not exclude possible specific or direct effects of indoleacetic acid and maleic hydrazide on resistance. Nevertheless, theoretically at least, the balance struck between the rates of supply, by synthesis and transport, of individual metabolites to the host cells at the locus of infection, and the rates of utilization of these metabolites required for maintenance of the host cells and of the parasite must exert a governing influence on the metabolism of the infections and the development of the parasite (see 24). Both the data presented here and Schwinghamer's results (22) make it difficult to dismiss the theory that growth regulating substances exert important effects on the development of obligate parasites, possibly through control of the direction and rates of transport of critical metabolites. Certainly, the elimination of competing growth centers in the host causes a breakdown in the resistance of some normally resistant varieties.

Acknowledgments

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THE PHYSIOLOGY OF HOST–PARASITE RELATIONS

IVA. THE EFFECT OF MALEIC HYDRAZIDE ON THE CARBOHYDRATE, NITROGEN, AND FREE AMINO ACID CONTENT OF THE FIRST LEAF OF KHAPLI WHEAT[1]

D. J. Samborski[2] and Michael Shaw

Abstract

Khapli wheat was grown in sand culture in 1-qt. crocks. Daily applications of maleic hydrazide (10 mg. per crock) were started about 10 days after sowing. After 5 to 12 or 15 days of treatment, samples were taken for analysis. Maleic hydrazide inhibited growth and caused increases in the dry weight, total soluble matter, sucrose, and free amino compounds, particularly glutamine and glutamate, in the first leaves. The treated leaves were also rich in starch which does not normally occur in the wheat leaf.

Introduction

Maleic hydrazide inhibits the growth and flowering of Khapli wheat, causes marked increases in dry weight per unit leaf area, and breaks the resistance of the plants to Race 15B of stem rust (7). The analyses reported here were carried out during the course of the work described in (7) and are indicative of the extent to which the metabolism of the wheat leaf is deranged by maleic hydrazide.

Methods

Little Club and Khapli wheats were grown in sand culture in 1-qt. crocks as described in (7). When the first leaves were fully emerged, about 10 days after sowing, the plants were thinned to five per crock and the daily addition of 10 mg. of maleic hydrazide per crock was started and continued for about three weeks.

Dry weights were determined after 12–18 hours in a forced-air oven at 95° C. The contributions of alcohol soluble and alcohol insoluble material to the total dry weight were determined after exhaustive extraction of the fresh material with 80% alcohol. Nitrogen, including nitrate nitrogen, was determined by a semimicro modification of the procedure given in (1). The dried tissue was boiled with 80% alcohol for two half-hour periods. The alcoholic extracts were decanted, combined, and evaporated under reduced pressure. The residue was taken up in distilled water. Aliquots of the aqueous solution were used for the determination of 'soluble nitrogen'. This fraction thus includes only the water soluble fraction of the alcohol extract. Dried samples of the alcohol extracted tissue were used for determinations of 'insoluble nitrogen'.

The free amino acids were extracted by blending approximately four grams of fresh leaves in 90% alcohol for 5 minutes in a Waring blendor at room

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temperature. The homogenate was allowed to stand for 24 hours at 0°– 3° C. The supernatant liquid was separated by centrifugation and the residue was washed once with 90% alcohol and recentrifuged. The alcohol was removed from the combined supernatants under reduced pressure at room temperature. The residue was taken up in distilled water, recentrifuged, and the supernatant once again evaporated to dryness at room temperature. The final residue was taken up in a convenient volume of 10% isopropyl alcohol at pH 6.5. These solutions were stored in the refrigerator and later subjected to two-dimensional paper partition chromatography, using first phenol water and then collidine–lutidine as irrigating solvents. The chromatographic techniques used were essentially those reported in References 9 and 10, and were developed for use in our laboratory by Dr. N. Colotelo (see 2 and 3). Despite the care devoted to the various stages of the analysis, the estimated concentrations of the individual amino compounds were not reproducible within ± 10%. Identification of the individual compounds was made by reference to 'maps' prepared by chromatography of mixtures of amino acids of known composition, and, where necessary, by co-chromatography of known compounds and leaf extracts.

Glucose, fructose, and sucrose were determined as described in (6). Duplicate determinations differed from each other by less than 5%.

Results

The ratio of alcohol soluble to insoluble matter for leaves treated with maleic hydrazide for 15 days was approximately double that for untreated leaves (Table I). After 12 days of treatment, 'soluble' nitrogen was nearly six times the level found in untreated leaves (Table II). The nitrogen content of the alcohol insoluble material was also increased by maleic hydrazide (Table II). The sugar analyses are given in Table III. On a dry weight basis, glucose and fructose contents were reduced by maleic hydrazide. Sucrose was markedly increased by 5 days of treatment but fell well below the level for the controls after 15 days of treatment. Starch analyses were not carried out, but iodine tests on cleared leaves showed that, by the 5th day of treatment, the treated leaves were very rich in starch. No starch was found in the control leaves and starch does not normally occur in wheat leaves, although phosphorylase is present (8). It does, however, occur at the loci of rust infections on susceptible species of wheat provided the illumination is not limiting (6).

After 5 days of treatment with maleic hydrazide, the leaves contained five times, and after 15 days of treatment, approximately 30 times the concentration of free amino compounds found in untreated leaves (Table IV). In the treated plants aspartic acid, asparagine, alanine, proline, and, particularly, glutamine showed marked increases from the 5th to the 15th day of treatment. By the 15th day, glutamine accounted for 4.8% of the total dry weight of the leaves. The accumulation of glutamine occurred at a time when sucrose content was declining (Table III). Between the 5th and 15th days of

treatment, appreciable decreases in serine and threonine and smaller decreases in glycine, lysine, and tyrosine occurred in the treated leaves.

Arginine, asparagine, glycine, proline, the leucines, lysine, tyrosine, and valine were not detected in the analysis for untreated plants. Other work (3) has shown that these substances are, as is to be expected, normally present in appreciable amounts in the wheat leaf. Failure to detect them here merely means that these substances were not present individually in amounts greater than approximately 0.001% of the total dry weight of the tissue. Of those amino compounds detected in the untreated leaves, only γ-aminobutyric acid

TABLE I

THE EFFECT OF MALEIC HYDRAZIDE ON THE PERCENTAGE DRY WEIGHT AND SOLUBLE AND INSOLUBLE MATTER IN THE FIRST LEAVES OF KHAPLI WHEAT

Days of treatment	Maleic hydrazide (10 mg. daily)			Untreated		
	% dry wt.	Soluble	Insoluble	% dry wt.	Soluble	Insoluble
5	14.85	—	—	12.84	—	—
15	26.87	0.6310	0.3690	14.58	0.4526	0.5474
16*	29.11	0.5875	0.4125	13.84	0.4116	0.5884

*Second leaves. Soluble and insoluble matter expressed as grams per gram total dry weight.

TABLE II

THE EFFECT OF MALEIC HYDRAZIDE ON THE SOLUBLE AND INSOLUBLE NITROGEN IN THE FIRST LEAVES OF KHAPLI WHEAT

Days of treatment	Maleic hydrazide (10 mg. daily)		Untreated	
	Soluble N	Insoluble N	Soluble N	Insoluble N
5	0.86	— (6.66)	0.55	— (5.58)
12	1.70	1.87 (5.07)	0.31	— (4.40)

NOTE: All figures as % of total dry weight except figures in parentheses, which give the insoluble N as a percentage of the dry weight of the alcohol insoluble matter.

TABLE III

THE EFFECT OF MALEIC HYDRAZIDE ON THE SUGAR CONTENT OF THE FIRST LEAVES OF KHAPLI WHEAT

Days of treatment	Maleic hydrazide (10 mg. daily)				Untreated			
	Glucose	Fructose	Sucrose	Total	Glucose	Fructose	Sucrose	Total
5	2.88	1.96	9.93	14.77	5.01	4.22	2.94	12.17
12	2.00	1.67	0.61	4.28	3.08	2.57	5.88	11.53

NOTE: Sugars as hexose % dry weight.

TABLE IV

THE EFFECT OF MALEIC HYDRAZIDE ON THE FREE AMINO COMPOUNDS IN THE FIRST LEAVES OF KHAPLI WHEAT

	% total dry weight			
	Maleic hydrazide*		Untreated	
Substance	5 days	15 days	5 days	15 days
Alanine	0.15	0.28	0.06	0.06
Asparagine	+	++	–	–
Aspartic acid	0.19	0.45	0.07	0.02
Arginine	Trace	–	–	–
Glutamic acid	0.76	1.03	0.31	0.09
Glutamine	0.37	4.80	Trace	–
Glycine	0.04	Trace	–	–
Leucines	0.04	0.06	–	–
Lysine	Trace	–	–	–
Proline	+	++	–	–
Serine	0.44	0.04	0.02	0.01
γ-Aminobutyric	0.07	0.10	0.01	0.03
Threonine	0.12	0.02	0.02	0.01
Tyrosine	0.02	–	–	–
Valine	0.05	0.12	–	–
Total	>2.25	>7.00	>0.45	>0.21

* 10 mg. per day.
\+ Present but not estimated quantitatively.
– Not detected but not necessarily absent.

increased during the period of observation. There was no change in alanine and moderate decreases occurred in aspartic acid, glutamic acid, glutamine, serine, and threonine.

After 7 to 10 days of treatment, glutinous droplets of fluid were secreted by the leaves. Some of these droplets were picked off with a dissecting needle and shown by chromatography to contain large amounts of sucrose, glutamine, and glutamate as well as smaller amounts of other amino compounds and glucose and fructose.

It is important to note that the analytical results presented here are expressed on a dry weight basis. The percentage dry weight of the leaves was not greatly increased after 5 days of treatment with maleic hydrazide but was approximately doubled after 15 days of treatment (Table I). It has already been pointed out (see 7, Table II) that about 75% of the increase in fresh weight of the treated leaves is accounted for by the rise in dry weight. When the increases in dry weight per unit area are taken into account, the increases in free amino compounds after 15 days of treatment become even more impressive. For example, in terms of the dry weight per unit area of the untreated leaves, the concentration of glutamine after 15 days of treatment would be at least double the value of 4.8% given in Table IV.

The foregoing results indicate that the metabolic disturbances which occur in Khapli wheat treated with maleic hydrazide are, in general, similar to those

which occur in other species (4, 5). These changes appear to result mainly from the continuation of photosynthesis after the inhibition of growth. The effect of maleic hydrazide in breaking the resistance of Khapli wheat to stem rust is discussed by Samborski and Shaw (7).

Acknowledgments

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THE ONTOGENY OF ADVENTITIOUS STEMS ON ROOTS OF CREEPING-ROOTED ALFALFA[1]

BEATRICE E. MURRAY[2]

Abstract

An ontogenetic study of adventitious stem formation on root segments of creeping-rooted alfalfa clones is presented. Unusual meristematic activity is evident first in the phellogen near a lateral rootlet. Continued activity in that region gives rise to a primordial dome from which adventitious stems eventually emerge. Concomitantly, in the subjacent phloem parenchyma cells dedifferentiation and subsequent redifferentiation into vascular tissues occurs. Thus a vascular system is formed which extends from the adventitious stems to the cambium region of the root and, in some instances, to the cambium of the lateral rootlet. Adventitious stems are initiated in secondary tissues of the root. Factors such as age of root, culture treatment, and inherent differences have an influence on adventitious stem initiation.

Introduction

A creeping-rooted alfalfa variety has been developed as a result of a breeding project at the Experimental Farm, Swift Current, Saskatchewan. The breeding history of this variety, named Rambler, was reviewed by Heinrichs (8). The present paper reports the results of an ontogenetic study to determine the origin and development of adventitious stems on the roots of this alfalfa variety.

Literature Review

Alfalfa plants which express the creeping-rooted habit of growth have been reported by a number of authors (6, 8, 11, and 16). Although Oakley (11) was the first to describe the morphology of this type of root system, the comments of the other authors are essentially similar. He noted the lateral spreading habit, frequent branching of the roots, and development of adventitious buds at intervals along the roots. Some of these buds became aerial shoots; others remained undeveloped for a time. The aerial shoots either developed a root system of their own or relied on the parent root for an indefinite period.

Creeping-rooted alfalfa is quite unlike the rhizomatous alfalfa described by Oliver (12). Rhizomes, stems of root-like appearance, are initiated only from

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the original root axis of the parent plant. These rhizomes extend laterally for varying distances, eventually root over part of their length, and their tips emerge from the soil as vegetative stems. Varieties of alfalfa with a rhizomatous habit of growth do not spread vegetatively to any marked degree under arid conditions (8).

Smith (15) observed a differential performance of varieties and strains of alfalfa in their ability to initiate adventitious buds on taproot segments. Buds could be induced to form only on clones of Ladak and *Medicago falcata*. He noted that age, length of root segment, and stage of development of the plant were important factors in adventitious shoot formation.

Adventitious stems occur naturally on roots of certain plants. This phenomenon was reported by Karpechenko (10) and others to occur in many of the F_1 plants of crosses between *Raphanus sativus* and members of the genus *Brassica*. The outgrowths on the roots from which shoots developed were described as exogenous in origin, consisting of parenchyma cells rich in starch and traversed by clusters of vascular tissue.

Wilson (20) noted adventitious shoot formation on roots of *Rorippa austriaca*. The shoot primordium was formed from cells close to the periphery of the root. The vascular tissue of this newly initiated shoot arose from the immediately subjacent root tissue. Siegler and Bowman (14) found the shoot primordium in 1-year-old apple roots to be almost invariably associated with the parenchyma rays. The shoot primordium was initiated in the ray cells in the outer phloem. Meristematic activity extended to the remainder of the phloem ray cells and even into the xylem. An apical meristem eventually was differentiated. Vascular connection between the meristem and the vascular tissues of the parent root was established by the differentiation of elongate meristematic cells internal to the shoot primordium.

Bud primordia may develop from almost any plant tissue depending on the parent species. According to Priestley and Swingle (13), such tissues may include the cells of the epidermis, chlorenchyma and collenchyma of the cortex, pericycle, periderm, and parenchyma of the xylem and phloem.

Wardlaw (18, 19) suggests that the apical meristem plays an important role in the development and differentiation of the vascular tissues of the shoot. He proposes that the meristematic potentiality of cortical cells, in this instance, is a residual property. Once growth is renewed in a detached meristem, the underlying cells may be affected by the inward diffusion of an activating substance. A somewhat similar suggestion was made by Ball (1, 2, and 3) in experiments on the shoot apex of *Lupinus alba*. Whether the apex was excised, or cut longitudinally into sectors, each segment regenerated an apex from a new group of initials. A meristem was differentiated between the new initials and the original procambium of the stem to either rings or rods of procambium. When leaves formed on the shoots, a normal eustele developed. He considered this phenomenon a downward differentiative effect of the stem tips, presumably caused by chemical substance proceeding from the apices.

Material and Methods

Three hybrids obtained from the cross of Ladak (*Medicago sativa*) and Siberian (*M. falcata*) were used as material for observing adventitious stem development on roots. Two of these, 24951 and 25029, were dense creepers (prolific adventitious stem producers) and the other, 25050, was a non-creeper.

All roots of 24951 produce adventitious stems. Under the conditions of culturing used in the experiment, lateral rootlets rarely were initiated. Therefore, 24951 was used for the ontogenetic studies of adventitious stem formation. To ensure genetic uniformity of all the root material used, the plants were clonally propagated by rooting stem cuttings. One root only was left on each stem cutting. Adventitious stems would not develop on root segments taken from young cuttings. For this reason, all root material used in the experiment was from cuttings permitted to grow for 12 or more weeks.

In order to stimulate adventitious stem development, the roots were cut into 15-mm. segments and suspended on thin rods in trays of running water in the light (Fig. 2). Strips of gauze placed on the rods served as anchorage for the segments and at the same time kept the roots moist. A water temperature of approximately 16° C. was observed to be satisfactory for stimulating stem development.

The portion of the root segment from which adventitious buds were observed to arise was prepared for ontogenetic observations. Carnoy's 3 : 1 was used as fixative and the material was then dehydrated according to the dioxane method reported by Walker (17) and embedded in Tissuemat, 60°–62° C. Safranin and haematoxylin were used in staining.

Observations

Adventitious stems generally arise on the roots of creeping-rooted alfalfa near a lateral rootlet region in plants growing under natural or laboratory conditions (Figs. 3, 4, and 5). To determine if the adventitious buds develop from preformed primordia, or if they are truly secondary, it was necessary to know the anatomy of the young root and observe the changes as it produced secondary tissues.

Root Development

Root Growth

The initial or primary root does not grow indefinitely as the main axis of the root system. Frequently one or two of the lateral roots branch off from the main axis. When this has occurred, the apex of the initial axis has sometimes ceased to function. The lateral roots in turn may branch. The degree of development and maturation along any root is influenced by this branching habit as well as by the environment in which the roots are grown. On the average, however, secondary growth is initiated 3 to 6 inches behind the root apex. Periderm formation begins at approximately the same time. At 6 to 10 inches behind the root apex, the periderm covers the root surface.

Root Anatomy

The primary root of creeping-rooted alfalfa resembles that of *M. sativa* as described by Hayward (7). It is characterized by a triarch, radial stele although tetrarch and diarch types are common. The number of protoxylem elements is limited to four or five per arc, with the metaxylem elements being centrally located. Groups of primary phloem alternate radially with these xylem arcs. Fibers differentiate in the outer primary phloem immediately adjacent to the pericycle, a uniseriate layer of cells. The endodermis has faint Casparian strips on its radial and transverse walls, but otherwise it resembles some of the innermost parenchymatous cells of the cortex. The root epidermis is a single layer of cells.

Secondary growth begins very early in the ontogeny of the root. The cambial zone, initiated between the primary xylem and phloem, becomes continuous over the protoxylem poles and soon forms a continuous cylinder of secondary tissues. The secondary xylem, in transection, consists of wedge-shaped arms of vessels, fibers, and parenchyma which alternate with wedge-shaped rays (exclusively parenchyma). The secondary phloem consists of sieve tubes, companion cells, fibers, and parenchyma. In the outer part of the phloem only, groups of fibers and starch-filled parenchyma cells remain. The rays are continuous from the protoxylem poles to the outermost part of the phloem. The alternate arrangement of the wedges of conducting tissue with the rays is much more distinct in the xylem than in the phloem (Fig. 6).

Periderm Formation

During the early stages of secondary thickening of the root, a series of tangential divisions in the pericycle indicates the beginning of periderm formation (4). The outermost layer becomes suberized before the endodermis, cortex, and epidermis are sloughed off. The phellogen, which gives rise to phellem on its outer surface and phelloderm on its inner surface, appears as a radially flattened layer in older roots (Fig. 7).

When the roots reach the stage at which adventitious stems can be initiated, a five or six layered periderm occupies the periphery of the root. The outer phellem cells are suberized. The phellogen is a layer of thin cells and the phelloderm may be one and in some instances two cells in depth. The phelloderm and the parenchyma cells of the xylem and phloem contain an abundance of starch. The phloem parenchyma retains its capacity for division and frequently the phelloderm and the smaller outer phloem cells are very similar.

Ontogeny of Adventitious Stems

At successive intervals of time in the ontogeny of adventitious stems there are certain changes which occur within the root during two intimately associated phases of activity. One phase is the initiation and development, at the periphery of the root, of a "primordial dome". (So called because organized primordia, or potential adventitious stem apices, occur among the

peripheral meristematic cells of the dome.) The other is the dedifferentiation of the subjacent secondary phloem parenchyma cells to form a "meristematic zone" in which vascular tissues differentiate and connect the peripheral primordia with the cambium of the root. Both phases are illustrated in Fig. 1.

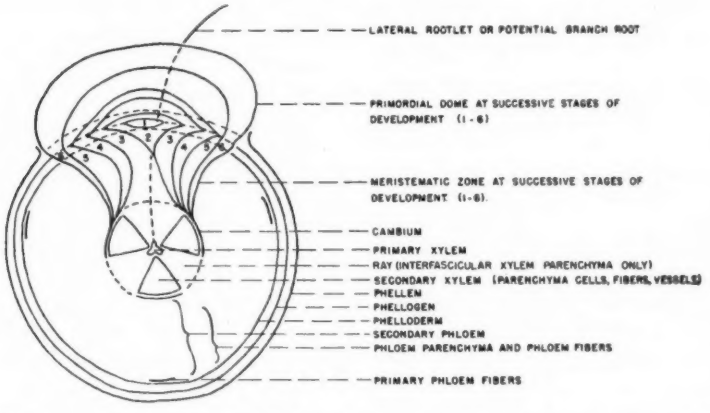

FIG. 1. A diagrammatic illustration of successive stages in the ontogeny of the primordial dome and meristematic zone.

Stage 1—initiation (1–3 days). Phellogen cells near a lateral rootlet begin to divide in tangential and radial planes indicating the initiation of the primordial dome with its adventitious stem apices.

Stage 2—formation and dedifferentiation (3–10 days). Additional phellogen cells and their derivatives continue their meristematic activity and the dome increases in size. Activity is initiated in the subjacent phloem parenchyma cells. They dedifferentiate and redifferentiate to form a meristematic zone which eventually extends from the peripheral primordia to the cambium of the root. Procambial strands are formed when adjacent phloem parenchyma cells of the meristematic zone divide in predominantly one plane.

Stage 3—development (10–15 days). The size of the primordial dome continues to increase and stem apices become evident. The meristematic zone continues to enlarge by the involvement of additional phloem parenchyma and interfascicular cambium cells. Vascular tissues begin to differentiate.

Stage 4—maturation (15–20 days). The domes reach their limit of lateral spread. Stem apices and their cell derivatives differentiate. Maturation continues in the meristematic zone.

Stage 5—stem primordia (20–30 days). Adventitious stems begin to elongate. Maturation of tissues in the stems, dome, and meristematic zone continues.

Stage 6—adventitious stems (over 30 days). From one to four stems continue to grow while others retain their rudimentary form until a later time. The vascular systems of these adventitious stems are continuous with that of the root.

Adventitious stem formation will be outlined and described according to the changes which occur in cells and tissues at different periods of time from the beginning of the culture treatment until adventitious stems form on the root segments.

Initiation—Stage 1 of Fig. 1 (1–3 Days)

Unusual meristematic activity is evident in some cells of the phellogen (Fig. 8) and its immediate derivatives. In this center of activity the nuclei of the cells become enlarged and some cells show signs of division in

predominantly a tangential plane. Initial meristematic activity generally is not confined to a single group of phellogen cells but involves two or three groups around a lateral rootlet region. The unusual activity in the phellogen is the initial stage of dome formation.

Formation and Dedifferentiation—Stage 2 of Fig. 1 (3–10 Days)

The original centers of unusual meristematic activity in the phellogen are extended laterally. Both radial and tangential divisions occur (Fig. 9). It is difficult to distinguish the groups of cells which function immediately as potential adventitious stem apices from the remainder of the meristematic cells of the dome.

Meristematic activity extends to the phelloderm and phloem parenchyma cells subjacent to the active phellogen (Fig. 10) and thus dedifferentiation is initiated in a basipetal direction. The dedifferentiation process is the initial stage in the formation of the meristematic zone. At this time, the parenchyma cells in the inner phloem region are not involved in the meristematic activity.

Usually at a lateral rootlet region, the phellem cells do not show a regular arrangement characteristic of the remainder of the root surface. The portion of any dome initiated immediately adjacent to a lateral rootlet likewise possesses irregularly arranged phellem at its surface. The phellem may be pushed out slightly by the meristematic activity of the subjacent cells.

In lateral rootlet regions where the domes are being formed, one or two conditions are evident at the periphery of the root. Either a small mass of proliferating cells is macroscopically visible at the surface of the dome (Fig. 10) or there may be no evidence of such cell proliferation. These conditions occur with equal frequency on roots placed under culture treatment. The two types of domes are essentially similar except for the initial surface proliferation during the early stage of development. Only observations on the performance of domes without proliferated cells will be described further.

The dome continues to enlarge by the division of cells derived from phellogen activity and by involving adjacent phellogen cells. The domes are usually three or four cells in height but their lateral dimension may vary.

The meristematic zone enlarges by involving additional phloem parenchyma in the dedifferentiation process until eventually the zone extends to the cambial region of the root (Fig. 12*c*). During the formation of the zone, some cells redifferentiate into procambial strands as a result of divisions occurring within adjacent cells in predominantly one plane (Fig. 11); others form smaller parenchyma cells. As soon as, and often before, primordia are visible at the periphery, procambial strands are evident in the zone.

The meristematic zone in transection (Fig. 12*a–c*), which is a tangential longitudinal section of the root, resembles that of a cone with its base at the periphery of the root (Fig. 12*a*), and the tip at the interfascicular ray cambium (Fig. 12*c*). The strands, which show a strong affinity for stain and occur in groups around the border of the zone (Fig. 12*b*), become continuous with the interfascicular ray cambium immediately adjacent to the vascular cambium of one wedge of conductive tissue (Fig. 12*c*).

Development—Stage 3 of Fig. 1 (10–15 Days)

The primordial dome is evident macroscopically as a transversely elongate, smooth-surfaced, green protuberance on the periphery of the root. The initial phellem cells flake off the surface of the dome.

Additional phellogen cells around the periphery of the dome become involved in its increase in lateral spread. Primordia of shoot apices are evident among the peripheral cells as organized centers of active development. Sometimes these units become evident as shoot apices even with a limited amount of dome formation.

It should be emphasized that, although shoot apices may be evident on a dome, shoot primordia at a much earlier stage of development are present among the remaining meristematic cells at the periphery of the dome. Additional development is required before these rudimentary primordia are distinguishable as shoot apices. Therefore, at any one time, primordia on the same dome are at different levels of development.

There are a number of changes in the meristematic zone. At the level of the cambium the zone extends across the ray to the wedges of conducting cells either side of the ray. The shape of the zone changes from that of a cone to either an open cylinder or (in transection of the root) a mushroom-like structure if there is a broad dome at the surface.

Xylem cells differentiate first midway between the peripheral primordia and the cambium of the root (Fig. 13). Xylem differentiation therefore is bidirectional (5). In general, at the conclusion of the period the meristematic zone has reached its maximum size, except perhaps at the periphery where some additional phloem parenchyma cells may dedifferentiate; all additional changes are those of maturation of cells already present.

Maturation—Stage 4 of Fig. 1 (15–20 Days)

The domes reach their limit of lateral spread. The primordia continue to differentiate and are evident as rounded apices among the peripheral cells (Fig. 14*a*). Some of the cells within the body of the dome develop into parenchyma cells and others become the procambial strands of the peripheral primordia. These strands are continuous with those of the meristematic zone. Thus there is a continuous series between the cambium of the root and the shoot apices which develop at the periphery.

Maturation continues in the series of strands which are in groups or clusters in a ring formation around the border of the meristematic zone (Fig. 14*b*). This formation resembles somewhat a cross section of a young stem. These clusters are continuous with the vascular tissues at the cambium region of the root (Fig. 14*c*).

Stem Primordia—Stage 5 of Fig. 1 (20–30 Days)

Stem primordia differentiate and eventually adventitious stems are produced. Although as many as 15 stem apices may be produced, usually from one to four continue to develop while the others retain their rudimentary form. Domes may show considerable height before adventitious shoots begin

to emerge at the surface (Fig. 15). Here, too, the adventitious stem primordia are at different stages of development.

Maturation continues in both the meristematic zone and the adventitious shoots which emerge from the dome. Certain clusters of vascular tissue in the meristematic zone are continuous with more than one adventitious stem on the dome.

It was stated previously that adventitious stems arise at a lateral rootlet region. For purposes of simplicity, many of the primordial domes used for illustrations contained no lateral rootlet. If a lateral rootlet is present the meristematic zone during dedifferentiation may become continuous with the cambium of the lateral rootlet, which has undergone some secondary activity, as well as with the cambium of the root. Subsequently, with the ultimate maturation of procambial strands in the meristematic zone and primordial dome, the vascular systems of the adventitious stems, the lateral rootlet, and the root become continuous.

On roots permitted to grow for a longer time than those used in the study of adventitious stem initiation, some primordial domes were evident as phellem-covered protuberances on the surface of the roots. Such domes developed to a certain level but remained dormant, or inhibited, and enclosed by three or four rows of phellem (Fig. 16). Subjacent to the phellem of such domes are meristematic cells or potential stem primordia. Groups or whorls of vascular tissue are evident in the central portion of the dome. There is no evidence of procambial strands connecting these whorls with the cambium of the root. However, after culturing the root segments with phellem-covered domes, adventitious shoots eventually emerged through the phellem. At this time newly differentiated vascular tissues extend from the whorls into the shoots that emerge from these domes and also to the cambium of the root.

Adventitious Stems—Stage 6 of Fig. 1 (over 30 Days)

Adventitious stems tend to emerge from the entire surface of the dome. The leaflets and stipules of three or four leaves generally form before the adventitious stems begin to elongate. The stem apex, which has a single tunica layer, is rounded and convex in shape. Leaf primordia arise along the flank of the apex. Leaflets form early in the development of the leaf. Two stipules differentiate at the base of the petiole.

Two axillary buds are initiated in the axil of each leaf. One is visible in the second axil and two in the fourth axil from the stem apex.

The stem has a eustele. As the tissues of the elongating stem mature, the interfascicular xylem becomes lignified and the parenchyma of the pith breaks down.

Generally the first leaf of the adventitious stems is not the typical trifoliate or monofoliate seedling leaf of alfalfa. Aberrant types are common and may include such anomalies as fused stipules, an extended petiole or blade, one or more leaflets on an otherwise normal petiole, and stipules absent from the base of the petiole. These aberrancies are restricted usually to the first leaf, all successive leaves being normal in development and appearance.

Performance of Root Segments of 25029

Although 25029 is a very dense creeper when grown under field conditions, root segments respond differently under culture conditions from those of 24951. Instead of a primordial dome, there is formed a large mass of cells (Fig. 17). These cells, which become vacuolate and somewhat enlarged, appear to be cut off externally by the phellogen. No primordia differentiate within the mass of cells and thus no adventitious shoots form on the root segments. Phloem parenchyma cells, subjacent to this mass of cells, do not dedifferentiate and therefore a meristematic zone is not initiated. This is the reverse of what was expected on the basis of the performance of intact roots under natural conditions of growth.

Performance of Root Segments of 25050

Under natural conditions of growth 25050 is a non-creeper and therefore it does not develop adventitious stems on its roots. If root segments of this plant are placed under culture conditions, large protuberances form but they remain enclosed within several layers of phellem (Fig. 18). Below the phellem of the protuberance or dome there are three of four rows of meristematic cells which cover its entire inner surface. Several whorls of vascular tissue differentiate within the dome. There is no evidence of adventitious stem initiation and no indication of meristematic activity in the subjacent phloem parenchyma cells although the roots remained in the culture treatment for approximately eight weeks. The protuberances appear to have been initiated by the phellogen.

Discussion

Ontogenetic studies have shown how, anatomically, mature roots of creeping-rooted alfalfa produce adventitious stems. Under culturing treatment, adventitious stems are initiated and developed by roots permitted to grow until the periderm, pericyclic in origin, consists of several layers of cells and the cambium has produced secondary tissues.

The phellogen shows unusual, but orderly, meristematic activity near a lateral rootlet region as the first indication of adventitious stem formation. This activity extends laterally to involve additional phellogen cells and in a subjacent direction to the phloem parenchyma cells.

The unusual meristematic activity of the phellogen and its daughter derivatives results in the formation of a primordial dome consisting of stem apices, all at different levels of development. From one to four of these stem apices continue to differentiate and produce the adventitious stems observed on roots of creeping-rooted alfalfa.

Accompanying the unusual meristematic activity of the peripheral meristems, or apices, there is an organized and orderly dedifferentiation and redifferentiation influence on the subjacent secondary phloem parenchyma. These phloem cells divide. Those which divide in predominantly one plane

form strands of elongate cells extending from the peripheral apices to the cambium ray cells of the parent root. With continued differentiation these elongate cells become vascular tissues. Thus the vascular system of the adventitious stems is continuous with the cambium and conductive system of the parent root.

Dedifferentiation of phloem parenchyma cells is evident during the early stage of unusual meristematic activity of the peripheral meristem and its derivatives. It seems evident that this activity exerts an influence on the subjacent cells causing them to respond in an organized fashion, a response similar to that observed (2, 19) in experiments with isolated apices. If a stem apex is required for the initiation of a subjacent affect on underlying cells, then not only do roots of creeping-rooted alfalfa contain a mechanism(s) for initiating stem apices but these apices are initiated at an early stage of dome formation.

Under culturing conditions, roots of both the creeping and non-creeping-rooted plants develop dome structures from the activity of the phellogen. However, in the non-creeper, in contradistinction to the creeper there is no initiation of a stem primordium or dedifferentiation of phloem parenchyma cells. The dome structure seems incapable of initiating organized redifferentiation and we find only scattered whorls of vascular tissue which show no pattern in their distribution. Roots of the two plant types have the potentiality to form vascular tissue. The difference lies in their ability to form meristems which would orient this vascular tissue.

The dome structure on the non-creeper is very similar to the dormant, or phellem-covered, dome of the creeper referred to previously (Fig. 16). In both, the vascular tissue remains unorganized and it may be inferred from this that only actively growing stem primordia can exert a directing influence on their vascular tissue. The inherent difference in creeping-rooted and non-creeping-rooted plants is in some way associated with the ability of the former to initiate, or create, adventitious stem primordia on all roots.

Creeping-rooted and rhizomatous alfalfa (12) are distinctly different in their growth habit. Rhizomatous alfalfa produces stems only from the original root axis of the parent plant. The spreading habit is brought about by rooting of the underground stems, or rhizomes. The spreading habit of creeping-rooted plants, on the other hand, is the result of adventitious stems being produced at intervals along all roots formed by the plant. A section of any root of the branched root system of creeping-rooted plants has the potential capacity to produce adventitious stems.

The capacity of roots of some creeping-rooted plants to produce adventitious stems can be upset by the culturing treatment of their roots. In cultured root segments of 25029, the phellogen produced a profuse mass of cells which continued unchecked resulting in the ultimate destruction of the segment. The proliferation is similar to that illustrated in Fig. 10, which is presumably an immediate response to the culture treatment. It has been suggested (9)

that the fate of a cell is limited in dedifferentiation by the presence or absence of various factors of its environment. If there is a mechanism controlling the balance between profuse proliferation and regulated growth and redifferentiation, it can be influenced markedly by the culturing of the root segments.

The formation of adventitious stems on roots is not unusual (13). Nor is it unusual to have this characteristic expressed in some of the progeny of a cross (10). The mechanism(s) responsible for adventitious stem initiation is still a mystery. The initiation of adventitious stems is no doubt an interrelationship of many factors. These could be physical (size and number of primordia), physiological (chemical stimulus for differentiation and growth and environmental responses), and genetic (an inherent characteristic of the plant). A knowledge of the non-genetic mechanisms which influence adventitious stem formation would be valuable to an understanding of the hereditary factors involved in the expression of the creeping-rooted habit in alfalfa.

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Explanation of Figures

Plate I

Fig. 2. The type of tray used and the method of suspending 15-mm. long root segments on gauze and thin rods. Water enters the tray through small holes in the plastic tubing.

Fig. 3. A root segment with six primordial domes, taken from the culture treatment. An adventitious stem is differentiating on dome 2 (arrow); none are evident macroscopically on the others. A lateral rootlet is visible near domes 2 and 5.

Fig. 4. A primordial dome from which an adventitious stem has emerged. Five stem primordia are visible.

Fig. 5. A portion of a root grown under natural conditions. Two adventitious stems are evident; others (black arrows) are emerging or are at an early stage of development (arrows extreme right).
(S—stem; R—root).

Plate II

Fig. 6. Transection of a mature root near a lateral rootlet region at a stage when adventitious stems readily are initiated (arrow). The secondary xylem has several wedge-shaped parenchyma rays alternating with vascular rays. The outer secondary phloem is predominantly phloem parenchyma interspersed with phloem fibers (circled). The periderm is 5 or 6 cells in depth. (45×)

Fig. 7. Longisection of the periderm prior to adventitious stem initiation. The phellem is three or four layers, the phellogen (arrow) a layer of thin cells, and the phelloderm one or two rows of cells in depth. The phloem parenchyma and the phelloderm contain an abundance of starch. (600×)
(PE—periderm; PM—phellem; PO—phelloderm; SPP—secondary phloem parenchyma; VR—vascular ray; PR—parenchyma ray; SX—secondary xylem).

Plate III

Fig. 8. Transection of a root showing the initial stages in primordial dome formation. Phellogen cells have divided in a tangential plane (arrows); in others the nucleus is enlarged. No changes are evident in subjacent cells. (600×)

Fig. 9. Transection of a root in which the phellogen cells have divided in both tangential and radial planes (arrows). Activity has extended to the phelloderm and some of the secondary phloem parenchyma (circled arrow). The phellem is irregular. (175×)
(PM—phellem; SPP—phloem parenchyma).

Plate IV

Fig. 10. Transection of a root in which there is limited proliferation of phellem-like cells external to the meristematic cells of the dome. The meristematic zone has extended into the phloem region (arrows). (150×)

Fig. 11. Transverse section of a root. Divisions have occurred within the phloem parenchyma cells in predominantly one plane to form the elongate procambial cells of the meristematic zone. One cell is in anaphase (arrow). (375×)
SPP—phloem parenchyma; PM—phellem; D—primordial dome.

Plate V

Fig. 12, *a–c*. Longitudinal tangential section of a root illustrating a transection of a primordial dome and meristematic zone. There is no lateral rootlet. At the periphery of the root (12*a*) the meristematic area is extensive while in the mid phloem region (12*b*) it is considerably smaller as few phloem parenchyma cells have dedifferentiated (arrow). At the cambium zone (12*c*) the ray cambium cells, adjacent to one of the arms of vascular cambium, are involved in the meristematic zone (arrow). (100×)
MZ—meristematic zone; SPP—phloem parenchyma; PR—parenchyma ray; VR—vascular ray.

Plate VI

Fig. 13. A longitudinal section of the root and a longitudinal section of the primordial dome through its shortest dimension. Vascular tissues have begun to differentiate. An isolated xylem strand (arrow) is evident. Procambial strands extend into the dome. The procambial strands of the meristematic zone are continuous with both arms of the vascular cambium (adjacent sections). (100×)

Fig. 14, *a–c*. Transection of a primordial dome and meristematic zone illustrating the arrangement of vascular tissues (longitudinal tangential section of the root). (105×)

a. Transection of the primordial dome with numerous clusters and branching strands of procambium within the parenchymatous tissue of the dome. Primordia are evident within some of the meristematic cells along the periphery (arrows). The vascular tissues of a lateral rootlet is in the center of the dome.

PLATE VII

FIG. 14, *b*. In the mid phloem region of the root large and small clusters of vascular tissue (arrows) are arranged along the border of the meristematic zone.

c. Near the cambium zone the clusters of vascular tissue are continuous with the vascular cambium in the ray cambium region of the root (arrows).

SPP—phloem parenchyma; MZ—meristematic zone; PR—ray; D—primordial dome; RV—lateral rootlet vascular tissue; PA—parenchyma of the dome; CA—cambium.

PLATE VIII

FIG. 15. A primordial dome with six adventitious stems visible in this plane (arrows) illustrates the arrangement of stems along the periphery of the dome. The entire surface area is involved in adventitious stem formation. (100×)

SPP—phloem parenchyma; D—primordial dome; MZ—meristematic zone.

FIG. 16. A primordial dome covered by four or five layers of phellem. Whorls of vascular tissue have differentiated within the dome. (150×)

PM—phellem; D—primordial dome; SPP—phloem parenchyma.

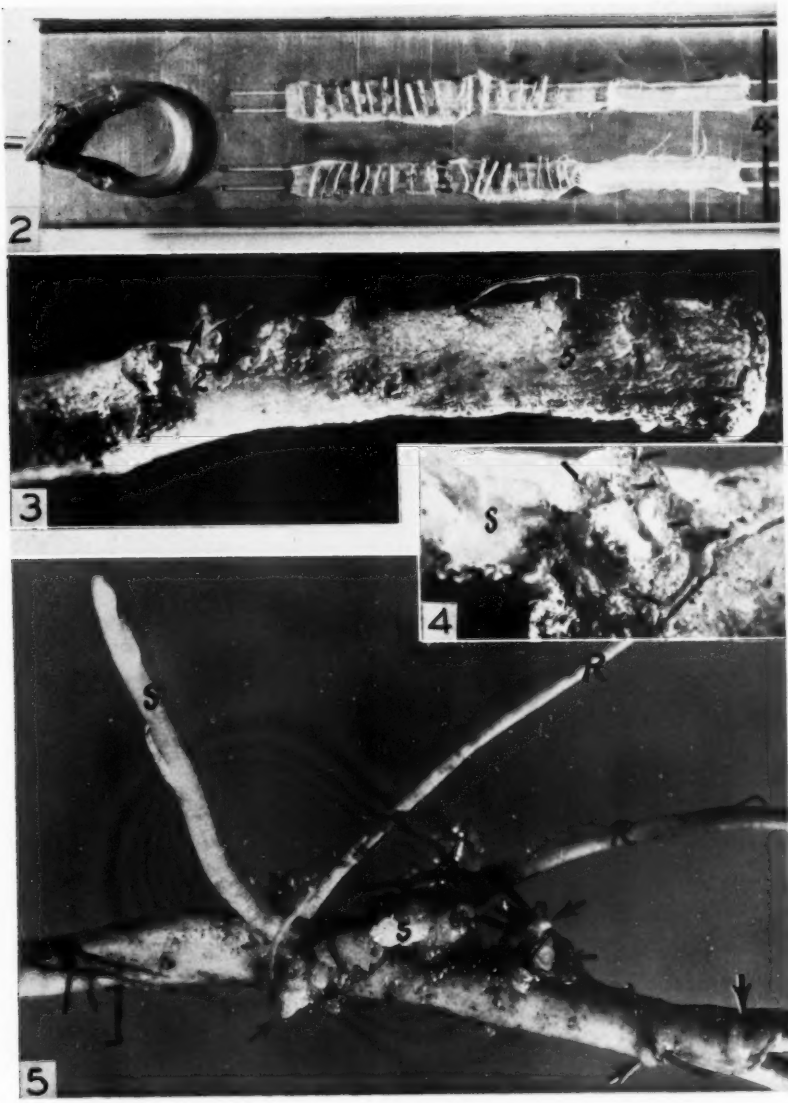
PLATE IX

FIG. 17. A longisection of a root illustrating the type of proliferation characteristic of root segments of 25029, when they are exposed to culture treatment. (55×)

FIG. 18. A cross section of a root illustrating the type of development on root segments of 25050, during culture treatment. Whorls of vascular tissue are interspersed within the ground tissue of the dome. The peripheral layers are meristematic. No stems differentiate. (60×)

SPP—phloem parenchyma; D—primordial dome; P—proliferation of cells.

NOTE: Figs. 2–18 follow.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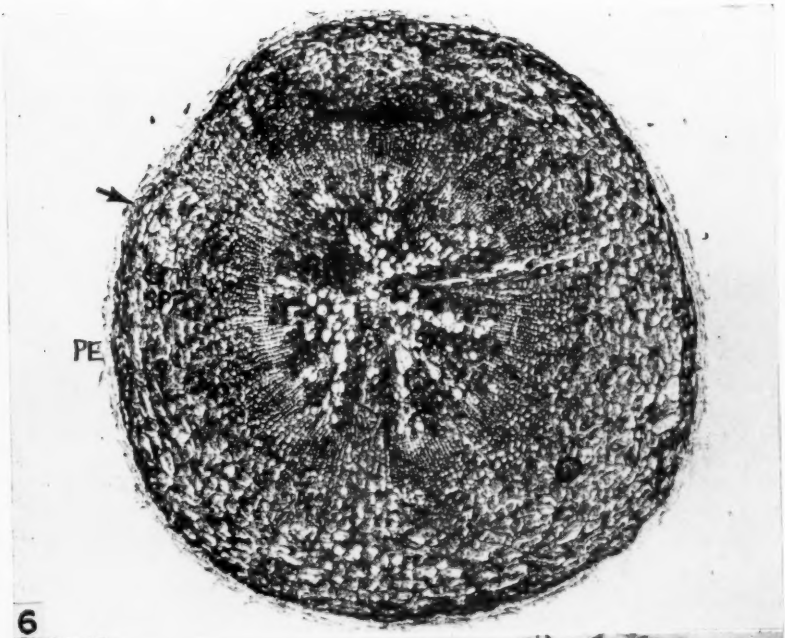
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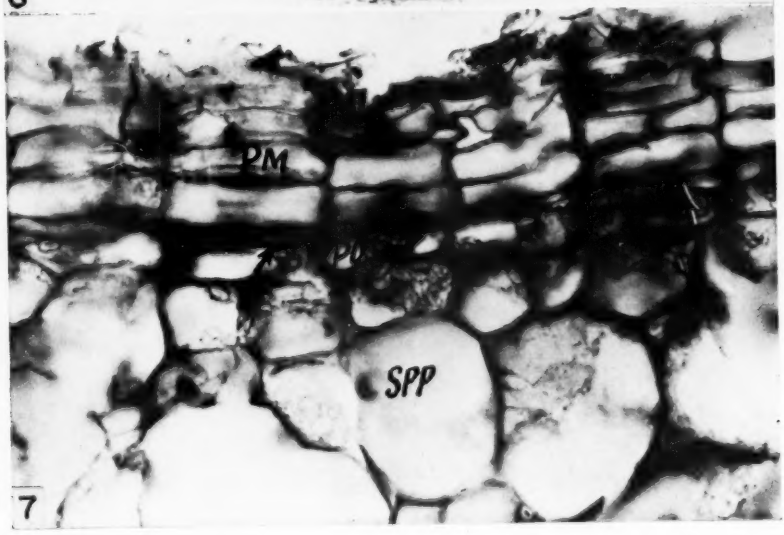
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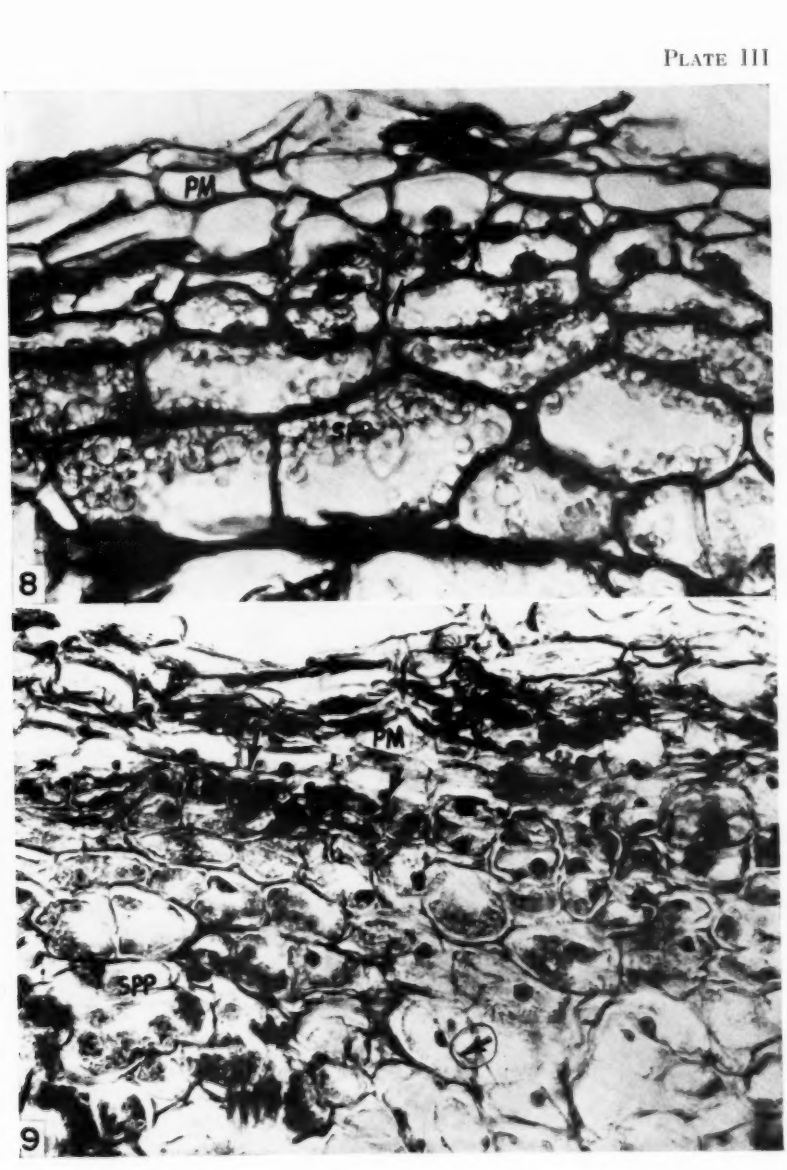
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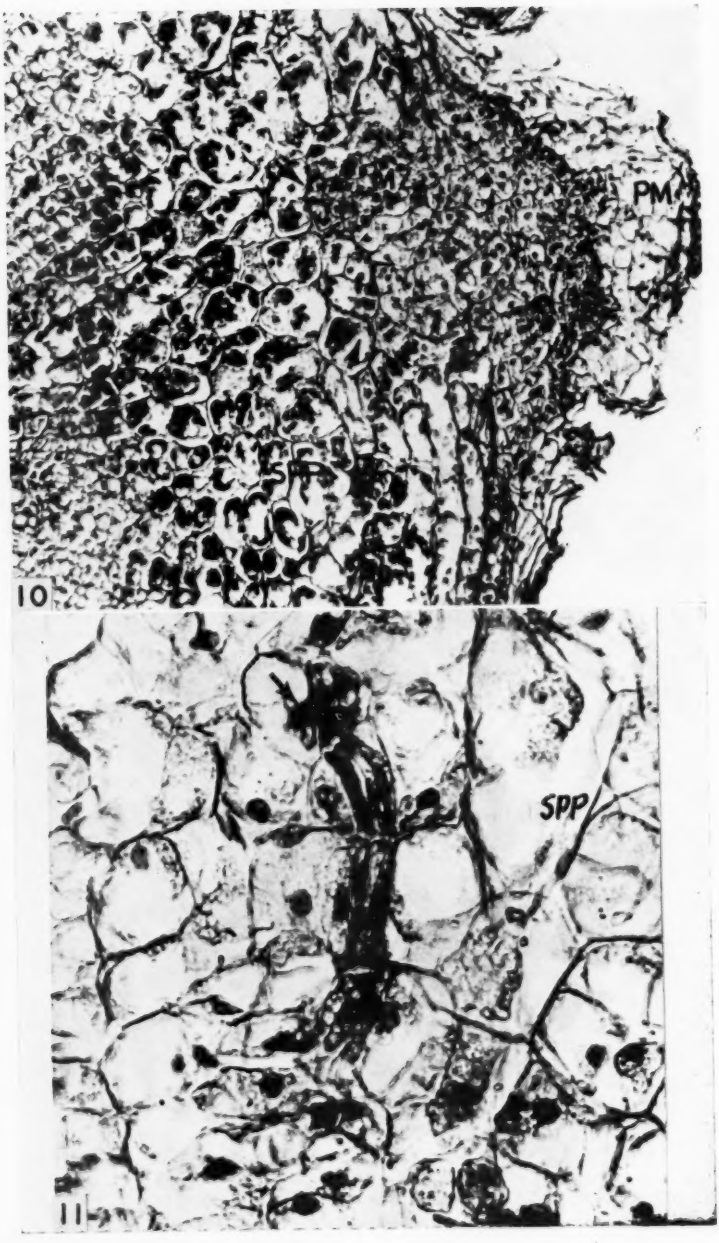
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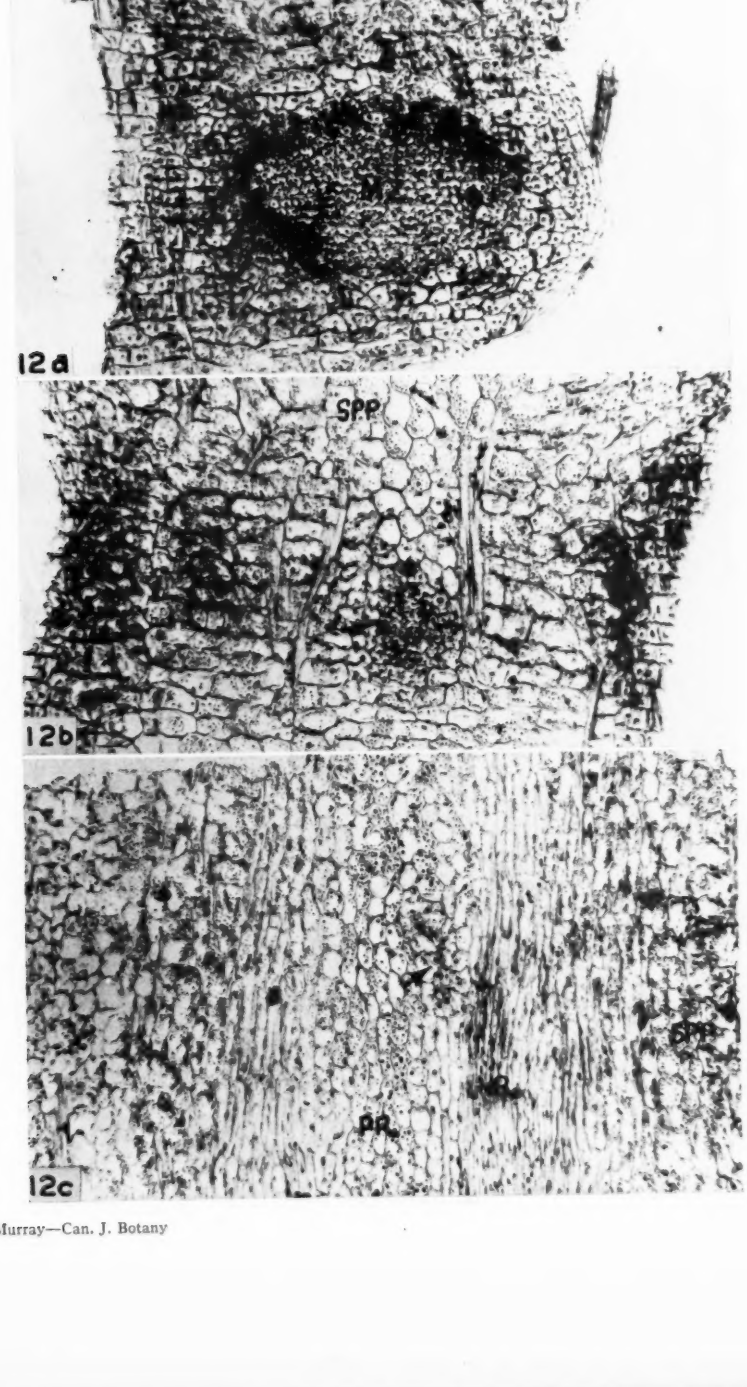

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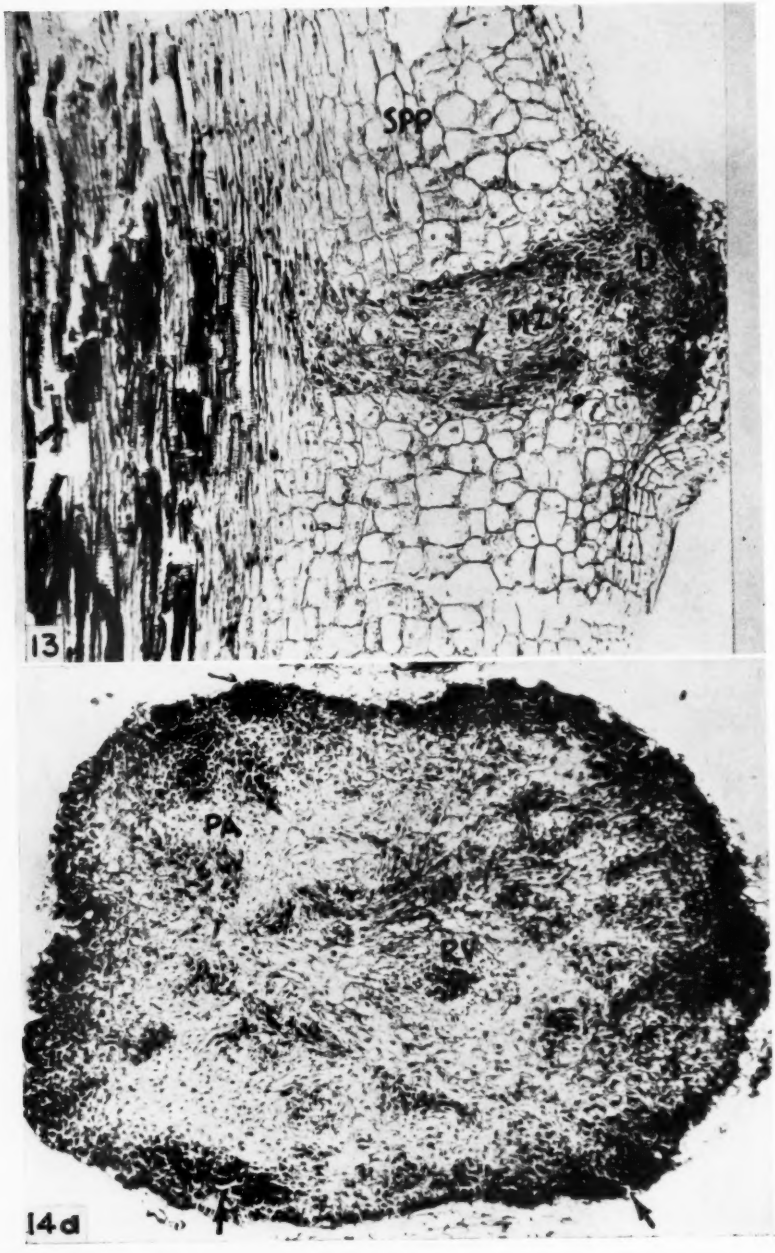

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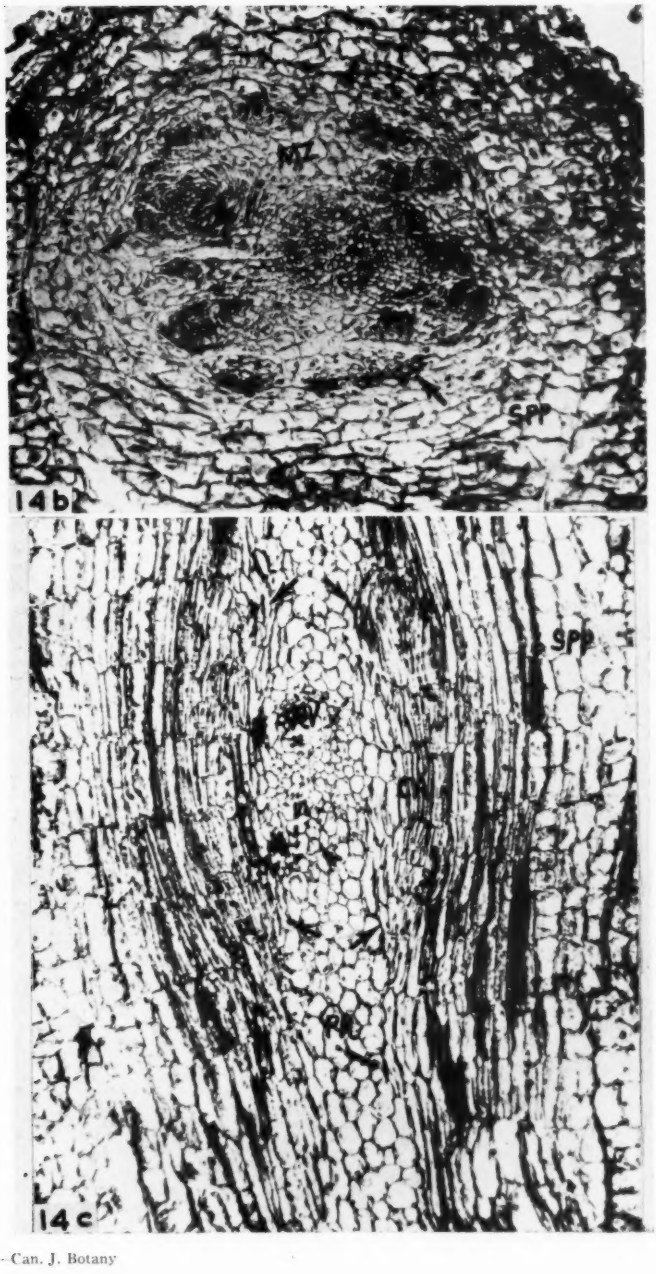

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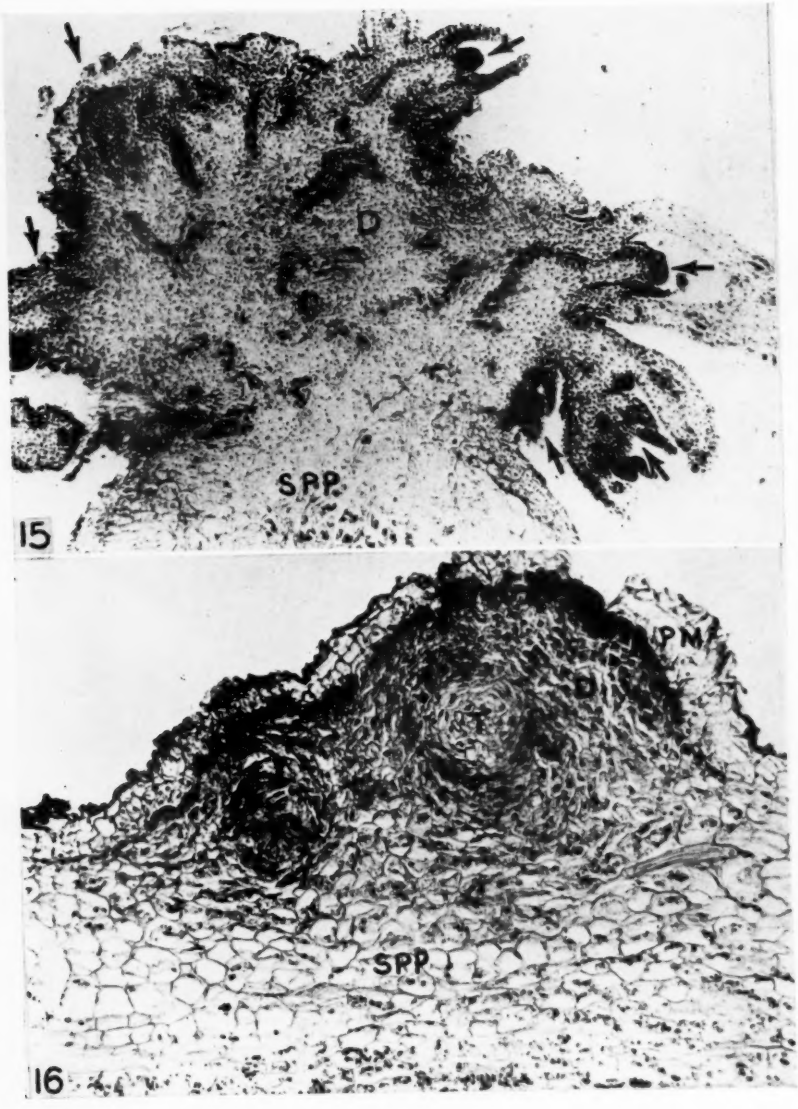
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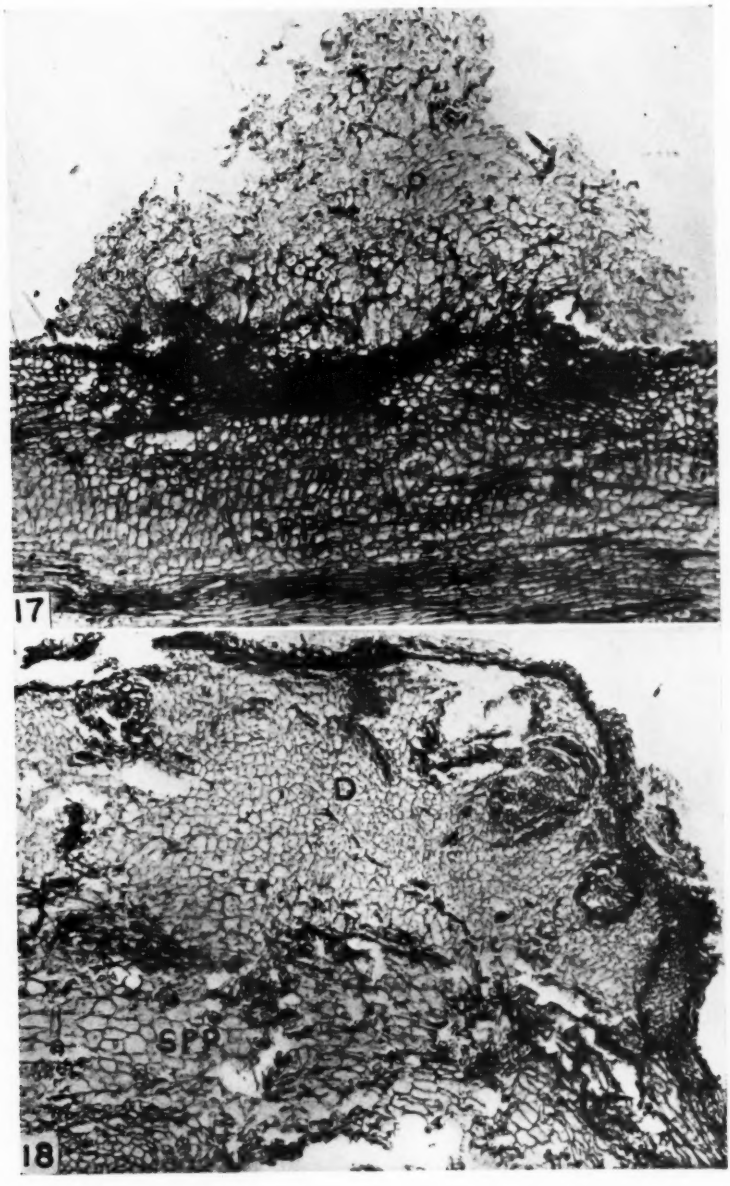
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STUDIES OF THE DEVELOPMENT OF ZOSTERA MARINA L.

I. THE EMBRYO AND SEED[1]

A. R. A. TAYLOR[2]

Abstract

Development of *Zostera marina* L. is described from proembryo to mature seed. The single cotyledon and the stem apex develop laterally from the nearly spherical young embryo. No radicle is produced; the hypocotyl develops into a swollen basal part (the original embryonic mass) and an elongated axial part formed by cell divisions at the base of the cotyledon and plumule. The cotyledon grows in part by cell divisions at its apex. Curvature develops in the axial hypocotyl mainly by unequal elongation of cells, whereas curvature of the cotyledon is mainly by unequal increase in cell numbers. A single procambial strand differentiates from the suspensor end of the basal part of the hypocotyl through the axial part into the cotyledon and plumule. The plumular stem apex develops a single-layered tunica over a several-layered corpus. It produces three leaf primordia, one with air-spaces organized, before dormancy sets in. Mid-vein procambium differentiates acropetally from the axial strand into each leaf primordium; lateral procambial strands differentiate acropetally and basipetally from near the base in each of the first two primordia. Two adventitious root apices differentiate external to the axial procambium at the base of the cotyledon. Seed coat development is described briefly. The nucellus remains a non-vascularized column of cells extending upward from the highly vascularized attachment region of the seed. The patterns of development reported support the interpretation that the starchy, shield-shaped main part of the embryo is the basal part of the hypocotyl and that the first leaflike structure is the cotyledon.

Introduction

Zostera marina L. is the best known, most widespread and probably most important of the few marine angiosperms. It is a monocotyledonous species, placed in the Englerian order Helobiae. It is usually grouped with *Potamogeton* in a family called by some Potamogetonaceae and by others Zosteraceae. While I was studying its growth and ecology in relation to the oyster industry of the Maritime Provinces, it became apparent that its morphology is unusual and remarkable. The opportunity was taken to collect material of *Zostera* for a study of its development.

There are many accounts of aspects of growth and development of individual species or groups of species of vascular plants, but integrated studies of the development of single species are rare. Miller and Wetmore (13, 14, 15) studied the developmental anatomy of the embryo, the seedling, and the apices of the mature plant of *Phlox drummondii* Hook., a dicotyledonous species. This represents the most complete available account of the develop-

[1]Manuscript received February 19, 1957.
Contribution from the Department of Biology, University of New Brunswick, Fredericton, N.B. This paper is from a thesis presented to the Graduate School of the University of Toronto, October, 1955, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2]University of New Brunswick, Fredericton, N.B.

[3]The term *saggital* will be used to designate the median longitudinal plane cut through the mid-line of the leaves; *frontal* will be used to describe the median longitudinal plane at right angles to this, that is, the median longitudinal plane cut parallel to the blades of the leaves.

ment of a single angiosperm species. Sharman (21) described the developmental anatomy of the shoot of *Zea mays* L. Others have provided information on this and other aspects of the development of *Zea*, yet no complete account is available although this is perhaps the monocotyledonous species which has been most studied.

This is the first of a series of papers which will describe and interpret those changes in external form and internal anatomy that occur during the development of *Zostera marina*. This paper covers development of the embryo and the seed; development of the seedling and the mature plant will be presented in subsequent publications.

The Proembryo

Gronland (9) described the gross over-all changes in the development of the embryo, but his interpretations of many structures are of doubtful value. Hofmeister (10) described pollination occurring by the pressing of the stigma into the open anther but later Delpino (4), Clavaud (2), and Engler (5) showed that in nature the stigmas of the pistils in a spadix become exserted through the opening of the sheathing spathe and are pollinated in the water by contact with the drifting masses of threadlike pollen grains. Cross pollination is therefore normal since the stigmas fall off before the anthers in the same spadix have opened (Engler (5), Dahlgren (3)).

Hofmeister's description (10) of the other processes leading to fertilization has been accepted. He described the normal growth of the pollen tube through the tissue of the style to the locule of the ovary where it grows, pressed close along the outer wall of the ovule, to the micropyle. The pollen tube bends sharply and passes through the micropyle to the apex of the embryo sac. At the micropylar end of the embryo sac are two synergids and a large egg nucleus. Rosenberg (18) described a single large central nucleus, whereas Dahlgren stated that in 15 cases examined there were two polar nuclei side by side. Three antipodal nuclei lie in a depression at the apex of the nucellus (columella). Actual fertilization has not been observed, but it is presumed to be double fertilization since one finds endosperm, consisting of cytoplasm containing numerous free nuclei (3, 18).

Hofmeister, Rosenberg, and Dahlgren each described the division of the zygote into an upper cell, which enlarges to become the giant suspensor cell,

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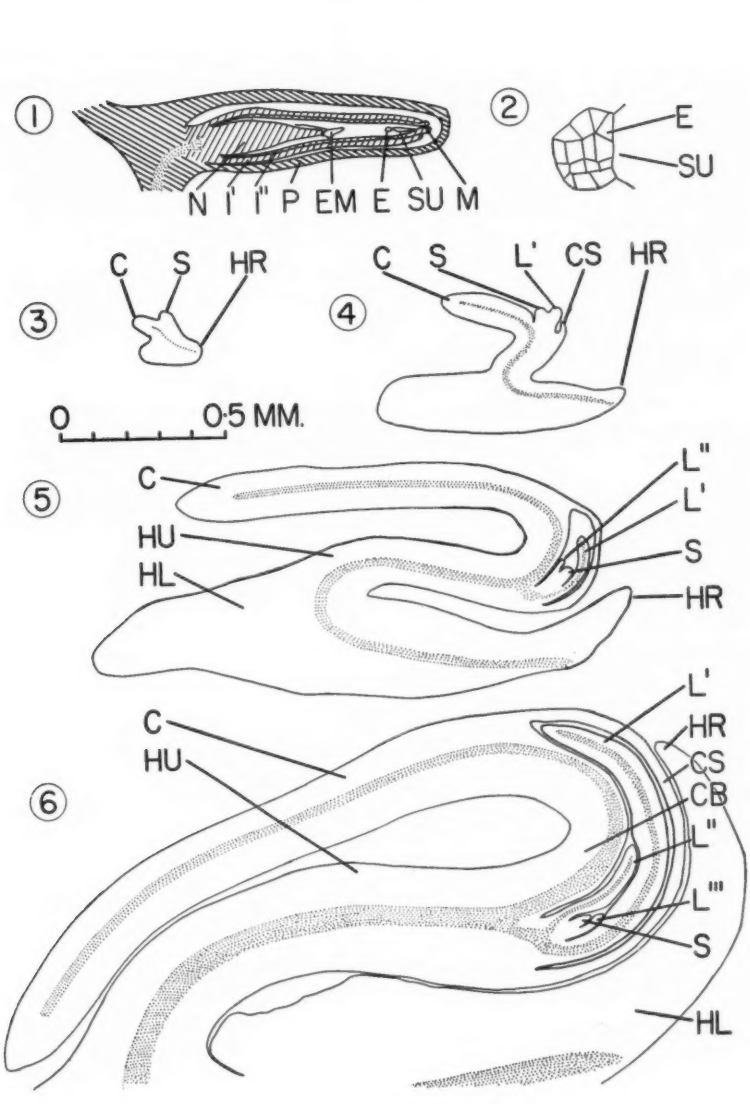
FIG. 1. Median longitudinal section of an ovary with small embyro near the right end. The style and stigma were to the left; point of attachment below, near the left (×15). *E*, embryo; *EM*, endosperm; *I'*, outer integument; *I''*, inner integument; *M*, micropyle; *N*, nucellus; *P*, ovary wall (pericarp); *SU*, suspensor.

FIG. 2. Very young embryo in median longitudinal section, redrawn from Rosenberg (18, FIG. 24); labels as in FIG. 1 (×275).

FIGS. 3–6. Median longitudinal sections of successively older embryos; procambium is dotted (×52).

FIG. 3. Very young embryo. FIG. 4. Young embryo, plumule not yet enclosed by cotyledonary sheath. FIG. 5. Embryo about one-third grown. FIG. 6. Part of a mature embryo. *C*, cotyledon; *CB*, base of cotyledon; *CS*, cotyledonary sheath; *HL*, lower part of hypocotyl; *HR*, 'radicle' end of hypocotyl; *HU*, upper part of hypocotyl; *L'*, *L''*, *L'''*, leaf primordia; *S*, stem apex.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N I' I" P EM E SU M
2
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C S L' CS HR
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and a lower cell which gives rise to the embryo. Hofmeister reported that the nucleus of the suspensor cell disappears, but both Rosenberg and Dahlgren observed that the nucleus remains. Fig. 10 shows the nucleus still present in the suspensor after the embryo has produced several hundred cells. The arrangement of the structures in the ovary at this stage is shown in Fig. 1. According to Hofmeister and Dahlgren the smaller lower cell divides longitudinally and then each daughter cell divides longitudinally at right angles to the previous division. According to Hofmeister all four cells now divide transversely to give an eight-celled embryo. Beyond this the sequence of cell divisions has not been followed. Johansen (12) assigned the embryogeny of *Zostera* to the *Ruppia* variation of the caryophyllad type on the basis of Hofmeister's description and comparison of it to that of *Ruppia*, but without reference to Dahlgren's work.

Dahlgren figured two isolated examples of further development of the proembryo. In the younger, an embryo of perhaps 30 cells (3, Fig. 3*a*), and in Rosenberg's Fig. 24 (18), redrawn here as Fig. 2, there is a suggestion of asymmetry at an early age. Rosenberg believed that his observations gave evidence that a curving in the development of the embryo gives rise to the cotyledon, stem, and hypocotylary axis and that Hofmeister had missed this curvature. It seems to me that Hofmeister's illustrations, Dahlgren's Fig. 3*b*, and the illustrations given here (Figs. 8, 9, 10, and 16) show that the cotyledon and plumular axis arise from a lateral (at first almost subterminal) extension of tissue. Rosenberg's Fig. 24 might be interpreted also as showing the beginning of such an outgrowth.

Goebel (8) gave a brief description of the "macropodous" embryo of *Zostera* and presented a figure of the proembryo at the stage where two small bulges appear on the side. He used this to support his interpretation of the large lower portion of the hypocotyl as a lateral outgrowth of the main axis, which shoves the axis to one side so that the "cotyledon no longer appears

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FIG. 7. Young proembryo in median longitudinal section; the suspensor is shrunken on the right (× 275).

FIG. 8. Ovary in transverse section; the developing seed contains a small embryo with beginning of embryo shoot on its lower right (× 90). *E*, embryo; *I'*, inner integument; *I''*, outer integument; *P*, ovary wall.

FIG. 9. Longitudinal section of part of an ovary; the embryo shoot is the bulge on the lower side of the embryo; endosperm cells appear above the embryo (× 90). *EM*, endosperm.

FIG. 10. The same ovary in median longitudinal section (50 μ after that in FIG. 9). Giant suspensor cell at right with embryo attached at left (× 90).

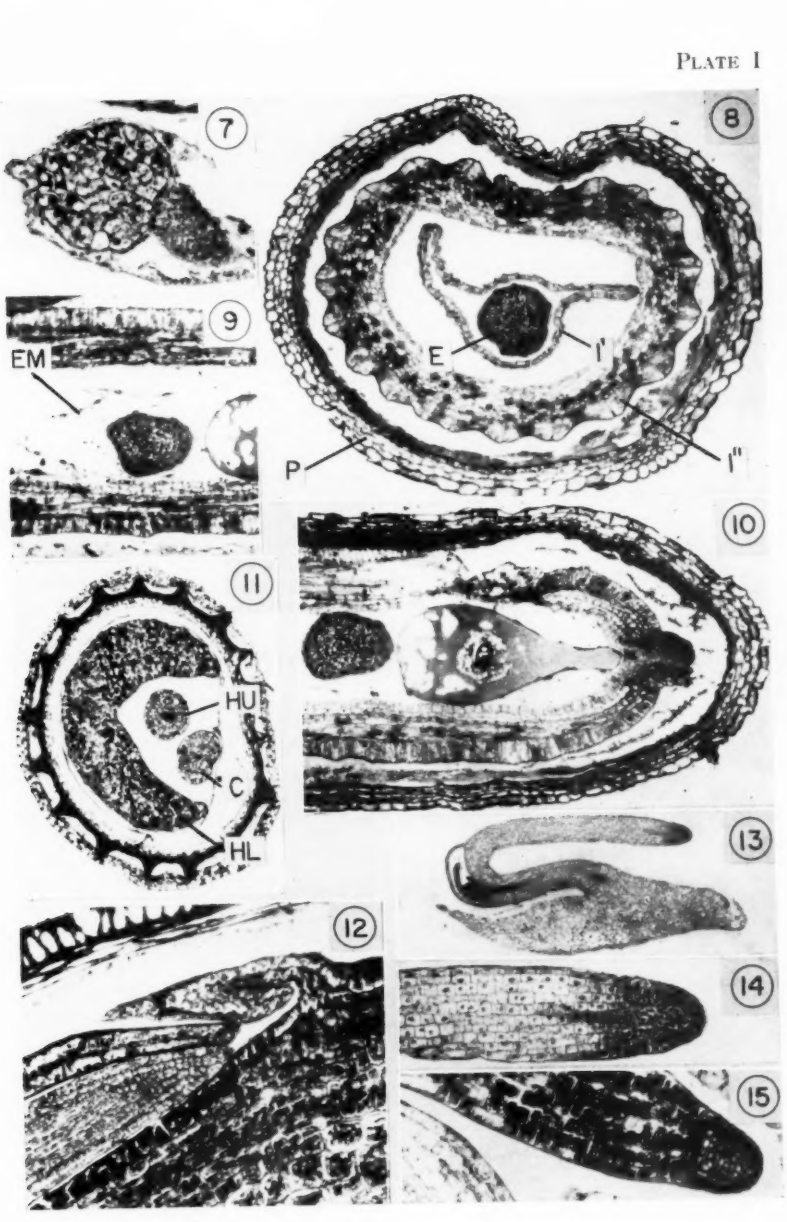
FIG. 11. Ovary with nearly mature seed in transverse section, approximately at level of *G* in FIG. 21; shieldlike part of hypocotyl to left, axial part of hypocotyl and cotyledon to right (× 30). *C*, cotyledon; *HL*, lower part of hypocotyl; *HU*, upper part of hypocotyl.

FIG. 12. Longitudinal section through opening of cotyledonary sheath at tip of first true leaf (× 190).

FIG. 13. Young embryo in near median longitudinal section (× 30).

FIG. 14. Growing tip of cotyledon from embryo in FIG. 12 (× 100).

FIG. 15. Cotyledon tip from a mature embryo showing group of degenerate cells after growth activity ceased (× 100).


7
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15

to be terminal". However, his labelling shows that he mistook the small protuberance which is the beginning of the stem for the cotyledon. Reference to Figs. 3–6 will show clearly that it is the protuberance farther from the suspensor which develops into the cotyledon.

Fig. 7 shows the nearly spherical form of the early embryo. Gradually a pronounced asymmetry develops with the extension of the plumular–cotyledonary bulge on one side. Figs. 8 and 9 show this development in transverse and longitudinal sections, respectively; the bulge is towards the lower side of the embryo in each photograph. In all specimens examined in section there was no evidence of a true bending of the embryo; rather a lateral expansion had developed by a local increase in cell number.

The Embryo

The individuality of the surface layer as a protoderm is established during the early stages just described (Fig. 8). A lateral bulge arises by means of anticlinal divisions at the surface, followed by anticlinal and periclinal divisions in the layers just beneath. As these continue, it becomes apparent that two separate lateral or subterminal structures are developing by a further localization of the superficial anticlinal divisions and the internal anticlinal and periclinal divisions.

The pattern of development in the young embryo can be seen in Fig. 16, a camera lucida drawing of a median longitudinal section of the young embryo sketched in Fig. 17, in which the individual cells have been outlined. The individuality of the surface layer is clear: it is produced and extended by anticlinal divisions of peripheral cells and apparently does not regularly contribute cells to the underlying layers. It may be thought of as a one-layered tunica. At this stage all of the cells are meristematic; the whole layer is protoderm giving rise to the epidermis.

The main mass of the embryo is below in Fig. 16, with the original point of attachment to the suspensor marked by an arrow. The 'bulge' mentioned before has developed above and has become divided into a larger and more actively growing protuberance to the left and a smaller one to the right. This smaller protuberance is the beginning of the stem apex and is the sole axial apical meristem of the embryo. It is marked *S* in Fig. 16 and is already elongating by apical growth at this early stage. It consists of a one-layered tunica, the apical part of the general protoderm, surrounding the beginning of a corpus which is now four to five cells broad. In Fig. 16, *C* denotes the upper actively growing protuberance—also elongating by apical growth—and this I consider as the cotyledon or first embryonic leaf. The structure 'below' this level is then hypocotyl and is designated in Fig. 16 as *HL*; *HR* is the end towards the suspensor and micropyle, the normal position of the radicle of the embryo.

As yet no clear procambium is evident, but the series of sections shows a form and orientation of cells as presumptive procambium extending from just above the point of attachment of the embryo to the suspensor, up the middle

of the structure, into the lateral expansion (shown above in Fig. 16), and towards both the apices. These cells are dotted in Fig. 16. Esau (6, Plate 84, Fig. 4) considered cells of similar appearance in the embryo of *Allium* to be procambium.

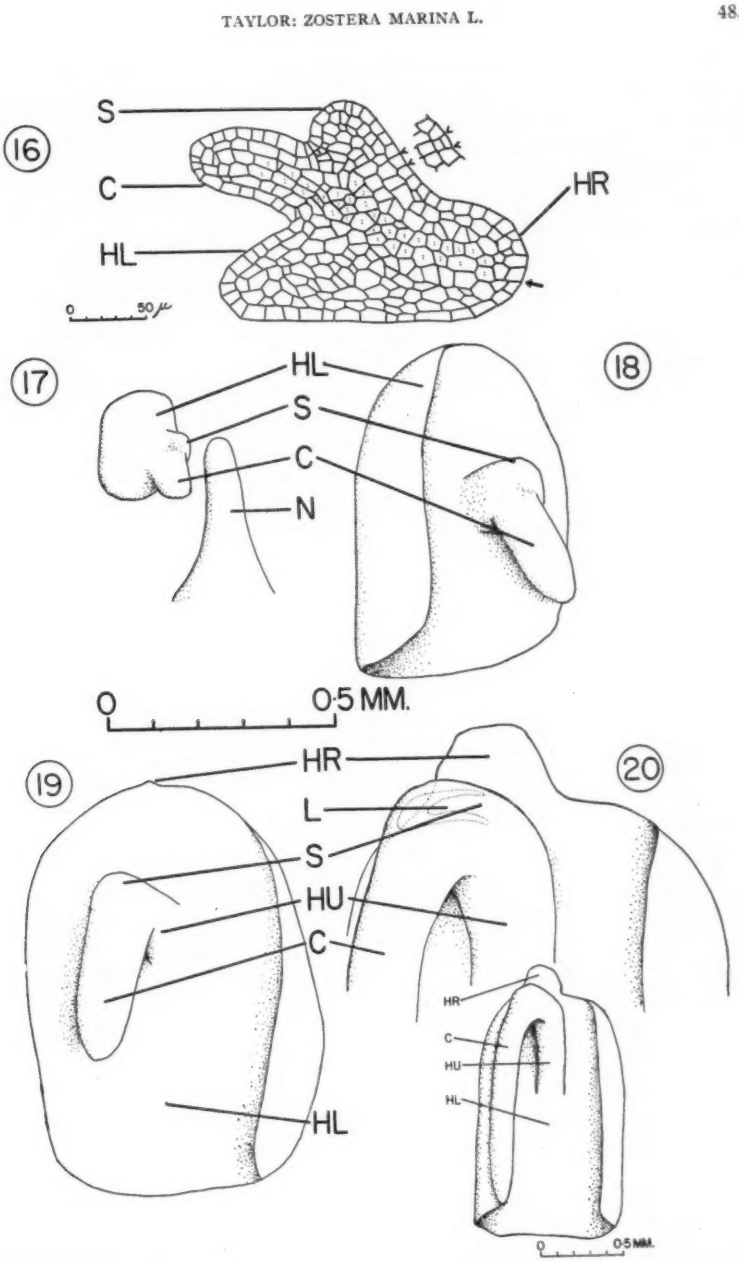
The Hypocotyl

The lower part of the hypocotyl grows by increase in number and size of cells. The form change is from a swollen disc or ellipsoid solid at the start into a swollen, expanded, shieldlike structure with a tab extending towards the concave side from the tip of the micropylar end. This change is illustrated in Fig. 18. The margins of the structure grow actively as it lengthens, bringing about its final shieldlike form. This was recognized by Hofmeister. As the structure increases in size, cell divisions become less frequent and are localized near the margins and later especially in the micropylar end where the "radicle" part of the hypocotyl develops. This meristematic activity had just become distinct in the embryo drawn as Fig. 4. In the embryo in Fig. 5 most of the cells present in the mature shield-shaped part of the hypocotyl had already been formed and growth beyond this stage (the embryo would double in length) is concerned with expansion of the existing cells. This was shown by counting the cells in the thickness and length of this lower hypocotyl. First, for thickness, cells were counted on a line directly across the hypocotyl from the concave surface to the convex one (in a position slightly to the left of the emergence of the axial portion in Fig. 5): the number of cells was 13 or 14 for both the younger embryo shown in Fig. 5 and for the mature embryo in Fig. 6. Second, for length, cells of the second layer beneath the epidermis were counted in the midline from a point about where the suspensor had been attached, to the end opposite: there were approximately 50 cells in the young embryo in Fig. 5 and 55 in the mature embryo in Fig. 6.

Gradually, as this expansion of the hypocotyl cells proceeds, the ground parenchyma and the epidermal cells become filled with round to oval starch grains 5 to 20 μ in diameter and with a prominent dark-staining hilum. The smaller grains occur regularly in the epidermal cells of the convex surface. There are other small granules present, mostly in the epidermis of the convex surface; they are probably protein. Starch was not found in embryos as large as that in Fig. 4; a relatively small amount is present in that illustrated in Fig. 5 but starch grains are abundant in the cells of the hypocotyl of larger embryos as in Fig. 28.

FIG. 16. Camera-lucida drawing of a young embryo in median longitudinal section. Dotted cells represent presumptive procambium; the arrow indicates the point of attachment to the suspensor. The small inset from an adjacent section shows the result of a periclinal division in the protoderm indicating the spread of the cotyledonary sheath around the stem apex (× 240). *C*, cotyledon; *HL*, hypocotyl; *HR*, 'radicle' end of hypocotyl; *S*, shoot apex.

FIGS. 17–20. Camera-lucida sketches of partly developed embryos dissected out of their integuments (× 70); the inset in FIG. 20 shows the whole embryo at a reduced scale (× 26). *C*, cotyledon; *HL*, lower part of hypocotyl; *HR*, 'radicle' end of hypocotyl; *HU*, upper part of hypocotyl; *L*, leaf primordium; *N*, nucellus; *S*, shoot apex.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The strand of procambium develops in the mid-line of the swollen hypocotyl starting just internal to the point of attachment of the suspensor as foreshadowed in the very young embryo (Fig. 16). Figs. 3 to 6 show the position of this developing strand and its extension into the cotyledon and plumule. It is formed as a compact cylindrical core of cells by cell divisions in planes parallel to the line of its development. Close to the attachment of the suspensor these cells remain little changed as the embryo matures. Distally, the procambial cells change gradually until in the mature embryo they appear as a rudimentary stele in which the vascular cells have undergone incomplete differentiation. In the center is a small group of from two to four cells which may be considered xyloic. Surrounding these is a mass of cells of which the innermost are, at some levels, large and expanded in the radial direction (5–15 μ × 8–20 μ in transverse section); the outer cells appear to have been formed by further divisions in the tangential and other longitudinal planes. These cells, probably phloic in nature, are not clearly organized in regular groups although at intervals around the central mass, cell divisions in the tangential plane give rise to smaller, radially arranged rows of two to four cells. The occurrence of these groups is not constant throughout the length of the 'mature' provascular strand of the lower hypocotyl. At different levels two, three, or four clear groups of such cells occur, and in some sections similar cells appear scattered in the general mass around the center.

The growth of the upper, axial portion of the hypocotyl takes place between the shieldlike part and the cotyledon above. Although no preparations made succeeded in showing clearly the cell divisions in this area, the small size of the cells, their general density, and the prominence of their nuclei combine to suggest a region of active cell division. This meristematic area lies just proximal to the point of emergence of the cotyledon and produces the main body of new cells which form the elongating, axial, upper part of the hypocotyl. It is probable that some residual cell divisions go on in the part of the structure which has entered into a stage predominantly of cell elongation.

To illustrate these changes a comparison has been made in Table I of the size and numbers of cells in the axial hypocotyl of the embryos illustrated in Figs. 4 and 5 and of one other embryo a little larger than that in Fig. 5.

Table I shows that when the hypocotyl of the young embryo was about half the diameter of the mature one it had its full complement of cells (or very nearly) in transverse section. Therefore, growth in diameter of the hypocotyl is by expansion of cells laid down in the initial extension of the axis.

On the other hand, elongation of the axial hypocotyl continues by increase in number of cells after the embryo is half grown, although not at the same rate in all portions of the axis. The number of epidermal cells increases three to four times while the number of subepidermal cells is not quite doubled; the average length of the epidermal cells decreases to nearly half while the size of the subepidermal cells is slightly greater than before. This is good evidence that anticlinal divisions continue in the epidermal cells after they have ceased in the cortex. The increase in number of cells in the cortex is due

TABLE I

COMPARISON OF SIZE AND NUMBERS OF CELLS IN THE AXIAL HYPOCOTYL STRUCTURE IN A YOUNG EMBRYO AND IN TWO MATURE EMBRYOS OF *Zostera marina*

	One-third to half grown embryo (*a*) (Fig. 13)	Mature embryos (*b*)	Mature embryos (*c*) (Fig. 6)
Longitudinal measurements			
(i) Plumular side of hypocotyl (outside curve)			
Length, μ	700	1450	1750
No. of cells in a longitudinal row of:			
(1) epidermal cells	23	90	100
(2) subepidermal cells	32	57	58
Av. length of:			
(1) epidermal cells, μ	30.4	16.1	17.5
(2) subepidermal cells, μ	22	25.5	30
(ii) Cotyledonary side of axial hypocotyl (inside curve)			
Length, μ	500	934	1250
No. of cells in a longitudinal row of:			
(1) epidermal cells	23	70	79
(2) subepidermal cells	30	54	50
Av. length of:			
(1) epidermal cells, μ	21.7	13.3	15.8
(2) subepidermal cells, μ	16.6	17	25
Transverse measurements			
Diameter of axial portion of hypocotyl at midpoint, μ	140	270	340
No. of cortical cells in radial direction:			
(1) to plumular side	3	3	3
(2) to cotyledonary side	4	4	4
No. of procambial cells in diameter of provascular strand	6 (approx.)	6–7	7 (approx.)

almost entirely to cells produced just beneath the cotyledon and plumule, whereas in the epidermis a large part of the increase is due to later divisions in the epidermal cells. In many places the conformation of the walls of the epidermal cells in the axial part of the hypocotyl of a mature embryo shows that four or more cells have arisen from one original cell by anticlinal divisions.

The stem-hypocotylar axis, growing out from the shieldlike part of the hypocotyl, is at first turned away from the suspensor (Fig. 16). Gradually, as the axial part of the hypocotyl becomes differentiated, a curve develops so that the axis points laterally away from the concave surface of the basal part of the hypocotyl (Fig. 4). As growth and elongation of the axial hypocotyl continue, the stem axis becomes turned back so that the plumule, sheathed in the base of the cotyledon, is turned towards the suspensor end. As the margins of the shieldlike part of the hypocotyl grow they enfold the S-shaped axial part of the embryo (Figs. 11 and 17–20).

Table I shows, by comparing the inside and outside of the curve of the axial hypocotyl as to length and number of cells, that in the younger embryo the curvature is due mainly to differential elongation of cells rather than to a greater number of cells on the outside of the curve. This is true for both the

epidermal and subepidermal cells not only in the case reported in Table I but also in the other embryos of similar size which were studied. In the mature hypocotyl, however, there is a substantially greater number of cells in the epidermis on the outside of the curve than on the inside although the numbers of subepidermal cells on each side of the curve are of the same order.

The growth to final form of the axial part of the hypocotyl is thus due first to cell divisions immediately behind the advancing plumule and cotyledon, and subsequently to an elongation of the cells formed. Since this elongation is greater on the side from which the cotyledon diverges than on the other, the axial part of the hypocotyl becomes bent back towards the suspensor end of the embryo.

The form of the procambial strand is similar to that described in the shield-like part of the hypocotyl close to the point where the axial part diverges. The cells show little differentiation even at maturity of the seed. Certain of the cells remain long; all are narrower than the surrounding cortical cells. They lack starch grains but some show a conspicuous mass of deeply staining material and others an extended lighter content with small granules which stain a dark red with safranin and crystal violet in Conant's quadruple stain.

The Cotyledon

In *Zostera*, the cotyledon has a very early origin, being initiated apparently at the same time as, but developing faster than, the stem apex of the embryo to form the asymmetrical bulge already described. At the same time as the hypocotyl grows, the cotyledon also increases in number and size of cells. It was mentioned earlier that the growth and curvature of the axial part of the hypocotyl causes the plumule to develop pointing towards the micropylar end of the ovule and brings the base of the cotyledon to that end. The tip of the cotyledon grows towards the opposite end and eventually lies next to the base of the nucellus (Figs. 3–6 and 17–20).

The mature cotyledon, like the leaves to be described later, may be considered as consisting of a *blade*, the distal part, a *base* beneath this, and a lateral extension of the base forming a closed *sheath* (Fig. 6). The first bulge of the cotyledonary primordium at the apex becomes the blade. The sheath appears by lateral growth of the margins of this structure to form a low collar surrounding the protuberance which is the stem apex. It has just begun to form in the embryo shown in Fig. 16. It grows upward by periclinal divisions of two rows of protoderm cells surrounding the base of the plumular apex. These divisions, which are anticlinal to the sheath itself, start at the edges of the primordium of the cotyledon and spread laterally until the sheath surrounds the developing plumule (Fig. 4). Its origin is entirely from the protoderm except where it expands into the main part of the cotyledon's base and also finally at its own basal junction with the axis where an incursion of one layer of cells occurs between its two epidermal layers.

It is clear that the basal sheathing part of the cotyledon is derived entirely from cells present early in development, between the levels of its divergence

from the axis and the top of the sheath; as a corollary, the distal part of the cotyledon is produced by cells above this level in the young developing structure.

The section drawn as Fig. 5 does not pass through the small opening (here 56 μ across) left at the middle part of the top of the sheath. A ridge of cells has developed across this surface of the cotyledon from one edge of the sheath to the other by periclinal divisions of the still meristematic epidermis. The structure so formed has the same position with reference to the cotyledon as the ligule has to the mature vegetative leaf of *Zostera* described by Sauvageau (19). At this stage it extends but three or four cells out from the surface of the cotyledon at the margins, and but two cells in the center.

This ridge of cells continues to grow forming a flap of tissue overlapping the sheath proper, becoming folded under (Fig. 12), and very nearly closing the sheath at maturity. A somewhat diagonally transverse slit finally results at the top of the cotyledonary sheath, just beyond the apex of the first true leaf of the plumule. In this example it was approximately 50 μ wide; the overlapping sheath and ligular parts give the slit a length of about 60 μ from the outside surface to the space within the sheath. This ligular flap is, like the sheath, two cells thick over most of its extent, with a thickness of three cells near its attachment to the blade.

In young cotyledons the apex of the 'blade' resembles a root tip, consisting of small closely packed cells in each of which the nucleus occupies a large volume (Fig. 13). Rows of cells extend back from these tip cells becoming: on the outside, the epidermis; inward from this, files of cortical cells; in the center, files of cells which undergo longitudinal divisions to become procambium. In older embryos the cells in the interior of the apex of the cotyledon appear to degenerate (Figs. 14 and 15) eventually becoming dead and empty.

The growth relationships between the epidermis and the interior cells and between the 'inside' and the 'outside' of the curve of the cotyledon are similar to what was observed in the hypocotyl. Some measurements are given in Table II of the distal part (blade) of the cotyledon and in Table III of the proximal basal and sheathing portion.

Table II shows that the main elongation of the cotyledon, as it grows to its final form in the mature seed, is by the continued production of new cells, not by continued elongation of the cells formed earlier. The cells of the subepidermis of older cotyledons are about the same size as those of younger ones. However, the epidermal cells continue to divide in the transverse anticlinal plane as the cotyledon grows until files of extremely short tabular cells are produced; these epidermal cells are shorter in older than in younger cotyledons. This is even more noticeable in the epidermis of the cotyledonary sheath and base (Table III).

The curvature of the cotyledon is the result of an increase in cell number on the outside of the curve as compared with the inside of the curve. The final cell length in the mature cotyledon was in most places less than in the

TABLE II

MEASUREMENTS OF NUMBERS OF CELLS AND LINEAR DIMENSIONS OF THE DISTAL PART (BLADE) OF THE COTYLEDON FOR FOUR EMBRYOS AT DIFFERENT STAGES OF GROWTH

	Embryos							
	Young (*d*)* (Fig. 4)	One-third to half grown (*a*) (Fig. 5)		Mature (*b*)		Mature (*c*) (Fig. 6)	
Longitudinal measurements							
Adaxial side							
No. of cells in epidermis	23 cells	33	(14)†	144	(30)†	170	(37)†
No. of cells in subepidermis	17 cells	50	(17)	98	(24)	104	(23)
Length of blade of cotyledon, μ	300	1150	(300)	1520	(300)	2000	(300)
Av. length of epidermal cells, μ	13	34.6	(21)	10.7	(10)	11.8	(8)
Av. length of subepidermal cells, μ	17.7	23	(18)	15.5	(12)	19.3	(13)
Abaxial side							
No. of cells in epidermis	19 cells	28	(10)	118	(26)	140	(26)
No. of cells in subepidermis	17 cells	49	(16)	83	(17)	100	(16)
Length of blade of cotyledon, μ	260	980	(260)	1350	(260)	1800	(260)
Av. length of epidermal cells, μ	13.7	35	(26)	11.4	(10)	12.8	(10)
Av. length of subepidermal cells, μ	15.3	20	(17)	16.2	(16)	18	(17)
Transverse measurements							
Thickness of blade (midway), μ	90	130		210		250	
No. of cells across cortex on side toward plumule	3 cells	3		3		3	
No. of cells across cortex on side away from plumule	4 cells	4		4		4	
No. of cells across procambium	4 cells	4		5		6	

*The letters (*a*), (*b*), and (*c*) designate the same embryos described in Table I; (*d*) designates an additional embryo, younger than the others.

†The figures in parentheses refer to measurements over basal portions of the blade of each of the older embryos equal in length to the whole structure reported for (*d*).

TABLE III

MEASUREMENTS OF NUMBERS OF CELLS AND LINEAR DIMENSIONS OF THE BASAL AND SHEATHING PARTS OF THE COTYLEDON FOR THE FOUR EMBRYOS REPORTED ON IN TABLE II

	Embryos			
	Young (*d*) (Fig. 4)	One-third to half grown (*a*) (Fig. 5)	Mature (*b*)	Mature (*c*) (Fig. 6)
Longitudinal measurements				
No. of epidermal cells along:				
(i) length of sheath	6–7 cells	46	208	226
(ii) length of abaxial surface of base	4–5 cells	8	61	54
Length of (i), μ	70	350	1220	1500
Length of (ii), μ	50	230	400	440
Av. length of cells of:				
(i), μ	10	7.8	5.9	6.6
(ii), μ	10	27.2	6.6	8.1
Transverse measurements				
Median thickness at midpoint, μ	75	110	160	190
No. of cells across the cortex at midpoint:				
(iii) toward plumule	1 cell	1	1	1
(iv) away from plumule	4–5 cells	5	5	5
No. of cells across the procambium	4 cells	5	5	4–5

same relative positions in younger cotyledons. Similarly, epidermal cells are shorter in the mature hypocotyl than in the young one, but in contrast, cortical cells are longer in the mature hypocotyl.

Early in the development of the cotyledon the single procambial strand originates by longitudinal divisions of the central cells, extending acropetally as the cotyledon elongates. In all embryos examined it reaches to the mass of small, dense apical cells. It consists of a compact core of cells very close to the plumular side of the cotyledon in the lower part (Table III), but nearer the center in the distal part. There are approximately 25 longitudinal rows of cells present in the lower portion. Fig. 21 is a diagram of a median longitudinal section of a three-quarter developed embryo and shows the cotyledon bent downward to the right; Fig. 22 is a photograph of a transverse section through this cotyledon at the level marked *A* in Fig. 21 and shows this vascular strand as it appears in the lower part of its course. The number of rows of cells which develop in the strand decreases towards the apex; 10 are present in Fig. 28 but further towards the tip only seven are present in Fig. 29. These procambial cells are narrow, have relatively dense contents, but lack starch grains; they closely resemble the procambium of the upper part of the axial hypocotyl. No clear characteristics of mature xylem or phloem were apparent.

Figs. 22, 28, and 29 show also the thickened outer walls of epidermis of the cotyledon. No chloroplasts were found in cells of the cotyledon, but starch grains are abundant in the cortical cells (Figs. 22 and 23).

The Plumule

I have described this structure as arising at a very early stage as a subterminal outgrowth of the hypocotyl; it continues the polarized growth evident then. The cotyledon may be thought of as the first leaf produced by the stem apex and soon outstrips it in growth. In Figs. 3 to 6 the sequence of leaf formation can be seen in relation to size of embryo. In Fig. 3 the embryo (also in Fig. 16) is about 0.2 mm. long and has the cotyledon well started; in Fig. 4 an embryo about 0.8 mm. long shows the primordium of its first true

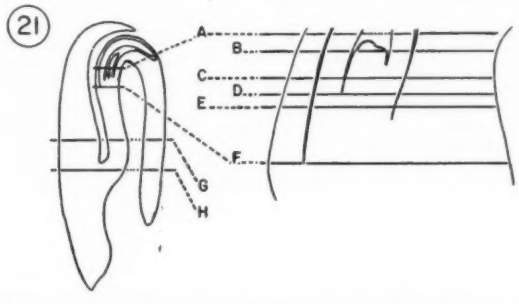


FIG. 21. Median longitudinal section of a three-quarter grown embryo to indicate the positions of the transverse sections shown in PLATE II, FIGS. 22–29 (× 15). The part between *A* and *F* is expanded on the left (× 125).

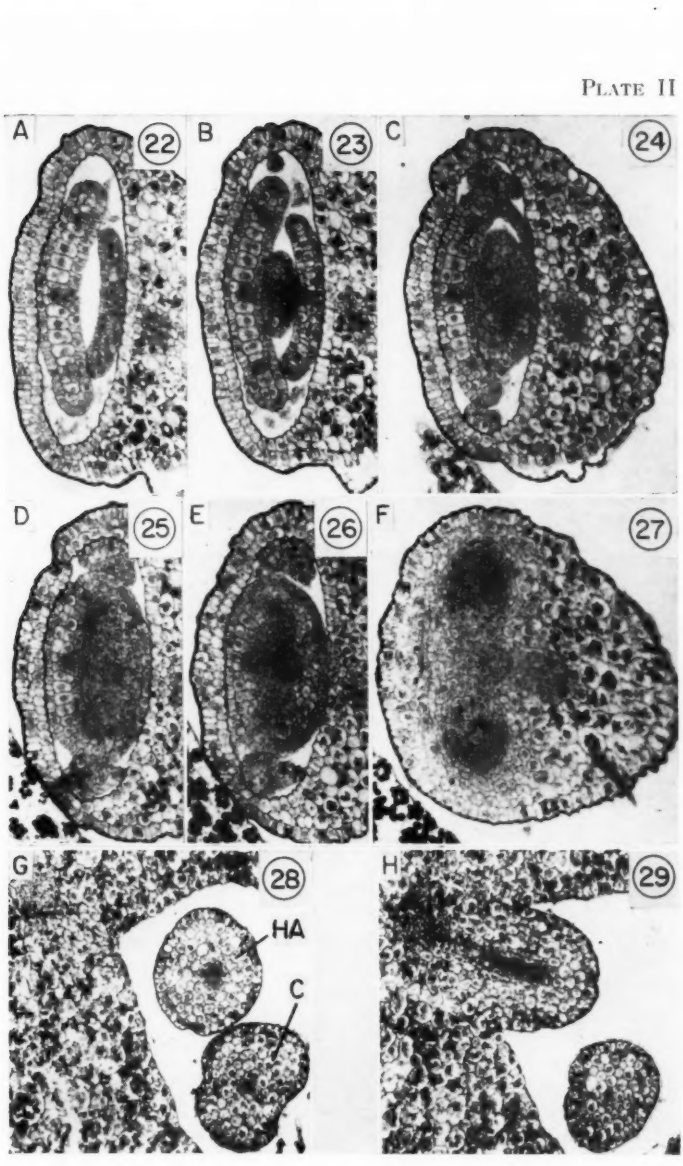
leaf well organized but not yet as long as the cotyledon in the previous example. The embryo in Fig. 5, about 1.6 mm. long, has formed the primordium of its second true leaf. Finally in the mature embryo, about 3 mm. long, the primordium of the third true leaf is present (Fig. 6).

No more than three leaf primordia have been seen in the fully developed embryos nor have branch primordia been observed although Hofmeister (10) stated that buds appear in the axils of the leaf primordia before the embryo is ripe. Possibly he mistook a leaf sheath in section for an axillary bud. The development of the apex and the leaves produced by it is continuous until the cotyledon and first three true leaf primordia have been formed. Activity then ceases until germination.

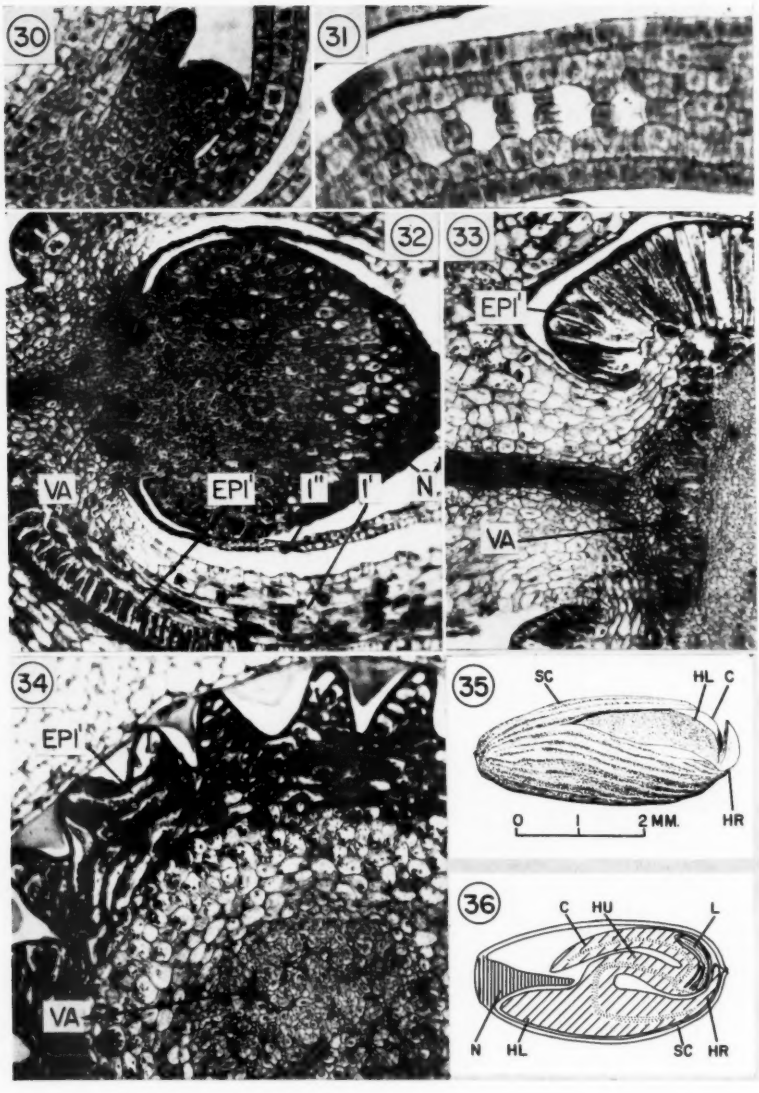
The young stem apex reaches its maximum size at the end of each plastochron. It has the form then of a rounded dome on an elliptical base. In its first plastochron (Fig. 16) it is four or five cells (42 μ) thick in the sagittal plane.[3] The slight bulge to the right at the tip of the apex in Fig. 30 together with the periclinally divided pair of cells in the outermost layer of the corpus show the origin of the third leaf during the fourth plastochron (the plastochron during which the cotyledon is formed being considered the first). There is also an appearance suggestive of a recent anticlinal division in the protoderm at the bulge. Here the apex is seven cells (42 μ) thick in the plane of the section (sagittal) and 10 or 11 cells wide (63 μ) in the plane at right angles (frontal).

The series of sections illustrated in Figs. 21 to 26 shows the degree of development in the plumule of an embryo which is probably three-quarters grown. In Fig. 23 the apex is cut at the level of divergence of the third leaf primordium (on the left). Again the apex is about seven cells across in the sagittal plane and about 11 cells across in the frontal plane. There has been little change in the appearance or size of the cells of the apical meristem; they are small cells in which the nucleus occupies a relatively large part and in which no large vacuoles are present. The protoderm cells are shorter in their tangential dimension than most of the cells of the interior although none are large.

The young leaf primordia are initiated by a locally increased frequency of anticlinal divisions in the protoderm, or tunica, accompanying a localized increase in number of periclinal divisions in the outer layer of the corpus immediately beneath. This occurs on the apex in the same longitudinal plane but above and on the opposite side from the previous leaf primordium. The increased meristematic activity spreads laterally around the apex as the primordium lengthens. The tunica layer seems to be involved only in the production of the dermatogen of the leaf. The periclinal divisions in the corpus give rise to the initial row of cells of the mesophyll; these divisions continue in the transverse plane of the developing leaf primordium. At this stage the whole primordium is meristematic, as is the apex from which it is developing. The leaf primordium is then, at first, an upraised tab of tissue at the margin of the stem apex, consisting of adaxial and abaxial dermatogen



FIGS. 22–29. Transverse sections to show the anatomy of a three-quarter grown embryo. Sections *A* to *F* (FIGS. 22–27) were cut from one embryo; section *B* was cut 21 μ below *A*; *C*, 35 μ below *D*; *D*, 28 μ below *C*; *E*, 14 μ below *D*; *F*, 70 μ below *E* (all 7 μ thick and stained with Conant's quadruple stain; × 155). Sections *G* and *H* were cut from another embryo of similar age (7 μ thick and stained with Foster's ferric chloride, tannic acid, and safranin technique; × 75). *C*, cotyledon; *HU*, upper part of hypocotyl.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layers with one meristematic layer between as shown by the youngest primordium in Fig. 30. Soon frontal (periclinal) divisions occur in the intermediate layer of cells at the base and continue downward in the cells of the stem widening it directly below each leaf primordium (Figs. 23–26).

The primordium grows first in length by transverse anticlinal divisions in these layers. Then longitudinal anticlinal divisions, apparently at the base, increase the number of longitudinal files in the mesophyll. At the same time similar divisions in the epidermis increase the number of its cells correspondingly. The epidermal cells remain narrow tangentially as a result of continued anticlinal divisions, whereas the majority of the internal cells commence to widen in this plane parallel to the surface of the leaf.

The beginning of midrib procambium can be seen while the primordium is still very young. It extends upward into the primordium from the acropetally developing strand in the axis (note the right hand primordium in Figs. 22 and 23). No procambium was apparent in the plumule of the embryos in Figs. 3 and 4, but in the embryo in Fig. 5 the median procambial strand was well differentiated in the first leaf primordium. In the mature embryo in Fig. 6 procambium was present and well advanced in both the median and lateral positions of the first leaf primordium and in the midrib of the second primordium; procambium had begun to form in the lateral positions of the second primordium too.

In the mature embryo in Fig. 6, and in another observed, presumptive procambium had extended up the center of the apex to within three or four cells of the tip. It has been formed by not more than one or two longitudinal divisions of the central cells, and its cells were not much longer than those of the surrounding corpus cells. This advanced differentiation so close to the tip of the apex probably resulted from cessation of growth before complete dormancy set in.

Once the leaf primordium has reached the size of the second in Fig. 23 (that on the right) it follows a regular pattern of development in which the fate of

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FIG. 30. Median longitudinal section through the shoot apex (plumule) of an embryo about one-third grown. The cotyledon and two leaf primordia are present (× 275).

FIG. 31. Longitudinal section of part of the first leaf of a mature embryo to show early formed air spaces (× 300).

FIG. 32. Base of young developing seed in median longitudinal section (× 100). *EPI'*, outer epidermis of outer integument; *I'*, outer integument; *I''*, inner integument; *N*, nucellus; *VA*, vascular anastomoses.

FIG. 33. Longitudinal section of base of a seed which contained a one-quarter grown embryo (× 100). Labels as in FIG. 32.

FIG. 34. Transverse section across region of anastomosing vascular tissue at base of seed which contained a three-quarter grown embryo. The vascular tissue is mainly xylem at this level (× 100). Labels as in FIG. 32.

FIG. 35. Sketch of a mature seed with the seed coat split open to reveal the embryo within (× 10). *C*, cotyledon; *HL*, lower part of hypocotyl; *HR*, 'radicle' end of hypocotyl; *SC*, seed coat.

FIG. 36. Diagram of a median longitudinal section through a nearly mature seed; procambium is stippled (× 10). Labels as in FIG. 35; *H*, upper part of hypocotyl; *L*, leaf; *N*, nucellus (columella).

most individual cells can be predicted. This second primordium is smaller in width and thickness (Fig. 23) and in length (Fig. 21) than the first. Also, there are fewer cells in its cross-sectional area, but there are more cells across the width of the young meristematic mesophyll than are to be seen in the first leaf primordium at the same stage. This appears to be caused by an increase in the width of the stem apex in the frontal plane as it develops in the embryo. A frontal section of a leaf primordium shows a continuous epidermis, increasing in number of cells longitudinally and tangentially by anticlinal divisions, and a series of longitudinal rows of internal cells which will produce the parenchyma, vascular tissue, and fibers of the mesophyll in the mature leaf. At the margins of the mesophyll are two or three rows of cells which undergo divisions in the flat plane of the leaf normally producing a group of small cells. In the second primordium in Fig. 23 (right hand side) a pair of cells newly formed by longitudinal anticlinal divisions can be seen next inward from the margin nearer the bottom of the photograph. These cells were beginning to form procambium of the lateral veins of this leaf. This development starts near the base of the leaf and extends acropetally into the leaf and, apparently, basipetally into the stem. This procambium was not present in the section shown in Fig. 22, cut 21 μ above that in Fig. 23, but examination of the sections in Figs. 23 and 24 showed several similar divisions presaging the development of procambium. In the older leaf (first primordium) these procambial strands were well developed and consisted of seven to 10 rows of procambial cells formed by periclinal divisions.

In the center of the second primordium in Fig. 22 is a group of four cells which have been formed by an anticlinal division of one cell followed by a periclinal division in each of the cells so formed. The pair towards the stem produces procambial cells and the parenchyma sheathing the procambium on the adaxial side; the other pair forms the parenchyma on the abaxial side of the procambium (note the median procambial strand of the first primordium in Fig. 23). Adaxial cells of the median procambial strand produce xyloic procambium while those to the abaxial side produce phloic procambium. There is so little differentiation of these cells in the embryo that it is not possible on the basis of form to designate these cells as precursors of proto- or meta-xylem or of proto- or meta-phloem.

Between the central procambial strand and the lateral strand on each side are the longitudinal files of somewhat larger cells destined to produce the main mass of mesophyll between the veins of the mature leaf. These cells enlarge in all dimensions until at the size of the first leaf primordium in Figs. 22 and 23 they begin to undergo median periclinal (frontal) division to form two cells across the thickness of the mesophyll. This is followed by a periclinal division of each daughter cell. The second leaf primordium usually develops this far before the seed becomes dormant. The first leaf primordium, however, develops much further. The cells formed towards the epidermis form a continuous subepidermal layer. Periclinal divisions of the inner daughter cells produce three to five cell layers across the thickness of the mesophyll between the subepidermal layers.

A separation of the cells in these inner layers occurs at this stage by the "rounding up" of their longitudinal anticlinal walls as noted by Sauvageau (19, 20) for the young leaves of mature plants. He described the formation of the schizogenous air spaces of the leaf mesophyll in the following way: a separation of the rows of the cells of the mesophyll takes place by a "rounding up" of their lateral (longitudinal, anticlinal) walls to produce small "rectangular" spaces at the corners (perhaps facilitated, it seems to me, by the previous periclinal divisions described above), and at the same time a daughter cell is cut off beneath each lateral wall. Thus, longitudinal rows of large cells are formed separated by two rows of small daughter cells. Between each successively higher placed pair of small daughter cells small air-spaces appear. These small daughter cells give rise to the transverse diaphragms.

Further cell division and enlargement of cells already formed lengthen and widen this first leaf primordium from this stage to that found in the mature embryo (Fig. 31). The original subepidermal cells divide anticlinally or obliquely anticlinally to increase the number of cells in the layer. At the same time the small cells which form the layers which separate one air-space from the next above undergo anticlinal longitudinal divisions and then enlarge. Thus, epidermis, subepidermis, and mesophyll are all increasing in the width of the leaf.

A few periclinal divisions may occur in the small cells 'roofing' the air spaces and in the original mother cells between the air spaces. This, with the increase in size of the cells, will bring about a thickening of the leaf primordium. The mother cells now divide frequently in the transverse anticlinal plane but not in the longitudinal anticlinal plane. They form the longitudinal walls partitioning the, as yet, small air spaces; the subepidermal layers, one on each side, produce the adaxial and abaxial walls of the air spaces; the small daughter cells roofing the air spaces form the transverse partitions, or diaphragms, by divisions in the periclinal and anticlinal longitudinal planes. In some places cells of the young diaphragms undergo transverse anticlinal divisions later to give a thickening in which the procambium of transverse anastomosing veins will develop.

The meristematic activity of the developing leaf primordium gradually extends laterally around the stem, as periclinal divisions of the surface layer of cells, to form the beginning of the leaf sheath at an early age. This development can be seen for the first leaf primordium in Figs. 24 and 25. The sheath was still very short here but had extended almost the whole way around the stem. The transverse sections photographed for these figures were somewhat oblique with the lower side in the illustration cut at a slightly higher level than the upper. By the time the embryo is mature the sheath of the first leaf primordium surrounds the stem apex completely and in five cells (22 μ) high on the side next to the mid-line of the cotyledon.

At maturity of the embryo this first leaf primordium is curved from base to tip and reaches to the small opening at the top of the cotyledonary sheath (Fig. 12). Measurements of one are: length 800 μ; width 350 μ at its mid-position; thickness varies, conforming somewhat to the mutual compression

amongst the structures within the cotyledonary sheath (Fig. 6). This thickness is 68 μ near the base beside the third leaf, 77 μ beside the second leaf, and 91 μ from just beyond the tip of the second leaf to a point 350 μ back of the apex of this, the first, leaf. From here it narrows gradually to a thickness of 64 μ at the position 94 μ from the tip to which it now narrows sharply. There is a well developed median provascular bundle and also a lateral provascular bundle on each side. Young, partly developed air spaces are also present occurring from the base to within 45 cells, 220 μ, of the leaf apex. The air spaces are separated by transverse partitions, four cells (approximately) along the direction of the width of the leaf and three to four cells across the thickness of the leaf, and by longitudinal partitions five to six cells deep and three cells across the thickness of the leaf. The second leaf primordium has attained a length of 325 μ and a thickness of 56 μ; it is eight to 12 cells thick through the median plane and six cells thick through the mesophyll between the bundles. The third leaf primordium is 84 μ long and has a thickness of 22 μ (three to four cells). Neither the second nor the third leaf primordium shows the beginning of air spaces. Fig. 31 shows air spaces in the first leaf.

Adventitious Roots

No primary root is formed in *Zostera*, but two adventitious root apices are present in the mature embryo. One develops on each flank of the central procambial strand that extends into the plumule from the hypocotyl at the level where this strand and that to the cotyledon diverge (Fig. 27). The root apices are organized by increased divisions in a group of cells, about four or five cell layers beneath the epidermis. These cell divisions do not seem at first to be in regular planes, but later it can be seen that anticlinal divisions in two layers of cells form two shell-like, hemispherical layers around a core of cells which by dividing both anticlinally and periclinally, push the apex of the root outward crushing adjacent external cells.

The 'roots' in the three-quarters grown embryo shown in Fig. 27 are approximately 65 μ long by 55 μ in diameter, whereas in a mature embryo they are about 150 μ long by 95 μ wide. These roots, about 16 cells in diameter, consist of three distinct layers: an outer one-celled layer, a 'cortex' of four layers of cells, and a potential central cylinder four to five cells across. No procambium differentiates in these roots in the embryo; however, they do show a relatively greater degree of development than is met in root apices associated with the fourth leaf from the apex of a mature vegetative shoot. The two root primordia never reach the surface in the embryo. The origin and development of the adventitious roots, the only true roots in *Zostera*, will be considered in detail in the description of the mature plant.

The Seed Coat

Gronland (9) showed figures of the external form of the two integuments during stages in their development but did not describe them or the mature seed coat. Hofmeister (10) described development of the two integuments

by active cell divisions at the apices of two crowns of cells elongating around the developing nucellus and eventually beyond it and the large embryo sac forming at its tip. Jensen (11) described the integuments in transverse section. The outer integument has a large-celled outer epidermis with raised radial longitudinal walls forming ridges down the seed coat; four or five layers of starchy internal cells lie between the outer and inner epidermises. The inner integument consists of two shrunken layers of cells.

In the maturing ovule, the outer integument has short outer epidermal cells of large radial and tangential dimensions (Figs. 8, 9, and 33). Midway down the length of a mature ovule the dimensions of these cells are: length 3–8 μ; radial thickness 16 μ in the middle, 32 μ at the radial walls; tangential width 36–90 μ. Fig. 8 shows the variation in the radial dimensions of these cells. The longitudinal radial walls grow faster than the transverse walls and faster than cell volume changes, with the result that the outer tangential wall of each cell is concave. These epidermal cells are aligned in 17 to 20 longitudinal rows and thus give rise to the 17 to 20 characteristic ridges present in the mature seed coat (Fig. 35). The seed coat reaches its mature size before the embryo has completed its development; it is nearly full size in Fig. 11. At maturity of the seed coat, the walls of this outer epidermis have become thickened, lignified, and hard. The dimensions of these mature cells are: length 12 μ, radial thickness 24 μ in the middle, 70–90 μ at the radial walls; tangential width 175–290 μ.

The other layers of cells of the outer integument are also complete by the time the ovule is mature but unfertilized. Four or five layers of cells lie between the two epidermises. These cells are much elongated in the long axis of the seed (65–160 μ), narrow in radial dimension (10–20 μ), and slightly broader tangentially (15–50 μ).

The inner integument consists of only two layers of cells (Fig. 8), as is characteristic of monocotyledons (16), except at the micropylar end. There it grows longer than the outer integument passing through the opening left at the apex of the latter to form a small cylindrical projection surrounding the very narrow micropyle (Fig. 10). It extends out for 72 μ and has about seven cells along its periphery to the apex. It is 90–100 μ across and has four to six cells in the radial direction extending from the micropyle to the periphery. The cells of the inner layer of the integument around the top of the embryo sac produce inward extensions appearing like droplets extending a short way (5–15 μ approximately) into the embryo sac.

As development proceeds, the inner integument becomes stretched and somewhat disorganized. The starch which both integuments have in their cells during early development of the seeds had disappeared in all mature seeds examined. Jensen (11) had reported starch present in the seed coat. Although the form of the two integuments in the ovule is close to that in the mature seed, they do undergo an increase in length and girth owing to increase in size of the individual cells. This results in considerable stretching and flattening of the inner cells of the outer integument, and in the stretching and

disorganization of the inner integument, which has become free from the outer. At maturity the outer epidermis and next two or three layers of cells of the outer integument show thickening and lignification as indicated by treatment with alcoholic phloroglucinol and concentrated hydrochloric acid.

The Nucellus

This structure remains as a column of cells extending up from the base of the seed, where the two integuments developed from it. The embryo develops around it. It can be seen in relation to the whole ovule in Fig. 1 and to the nearly mature seed in Fig. 36. The nucellus itself is not vascularized, but a single compact ovular vascular strand extends to its base as an extension of the vascular bundle of the carpel (Figs. 1, 32, and 33). This strand consists of a group of three or four longitudinal rows of annular or scalariform-thickened tracheary elements surrounded by phloem. It ends at the wide base of the nucellus in a series of radially expanded anastomoses, proximally of phloem and distally of xylem. The general position of these anastomoses can be seen in Figs. 32 and 33; the radial anastomoses of the xylem are shown in Fig. 34. The nucellus extends beyond as a cone of parenchyma cells reaching full development before fertilization of the egg. The outer epidermis of the outer integument extends slightly basally around the funiculus by a great increase in height of its cells (Fig. 33) together with the greater increase in diameter of the ovule beyond the base. As the seed matures, the nucellus assumes a rather shrivelled appearance.

There is no appearance of an abscission layer as such; rather, abscission of the seed from the funiculus seems to take place as a result of the death of this tissue as the seed ripens and the spadix becomes senescent.

The Seed

The seed is shed at maturity by a splitting of the unilocular ovary along its abaxial surface. Tutin (22) believed that this splitting was initiated in the ripe ovary by the formation of a bubble of gas from photosynthesis.

The seeds are heavier than the sea water and sink to the bottom where they may be buried in the soil or moved away by wave action. Seeds from Prince Edward Island waters (Fig. 35) are oblong, slightly concave at the basal or attachment end (by reason of the downward extension of the outer epidermis of the outer integument), and rounded at the micropylar end with the protruding inner integument forming a slight projection there. The mean dimensions for 138 seeds measured were: length, 3.38 mm. (s.d. ± 0.20 mm.); diameter, 1.46 mm. (s.d. ± 0.12 mm.). The surface shows usually 17 to 18 longitudinal ridges, occasionally as many as 20, with one or two extending but part way down the length. The color ranges from olivaceous or grayish to dark brown but there is a lighter, almost straw-colored, narrowly elongate, triangular streak extending from the micropylar end about half way down the surface. This streak represents the lighter colored cotyledon showing through the translucent seed coat.

The embryo within fills almost the entire space, the great bulk being the expanded lower part of the hypocotyl. In many seeds this grows to fill all of the space within the seed coat except for the part occupied by the hypocotyl–plumule axis and the columnar remnant of the nucellus.

Discussion

Because the form of the embryo of *Zostera* is unusual, its structure has been interpreted variously. Here, we have called the enlarged, shieldlike structure the "basal part of the hypocotyl" in agreement with Jensen (11), Raunkiaer (17), Rosenberg (18), Goebel (8), Arber (1), and Dahlgren (3). Gronland (9) and Tutin (22), however, considered this structure to be the cotyledon or scutellum. Fernald (7) described the cotyledon as obsolete—a rather curious designation from either of the above points of view. Hofmeister (10) called this shieldlike part of the embryo the "axis of the first order", and the remainder of the embryo an "axis of the second order" arising laterally from it.

The three ways in which the embryo has been interpreted might best be stated as follows:

(1) The whole shieldlike structure is the cotyledon with the remainder of the embryo growing laterally from its concave surface. This was the view of Gronland (9). All the evidence is against this interpretation: the structure called by him cotyledon is basal, organized at the point of attachment of the embryo to the suspensor; after germination it produces expansions of the epidermal cells of the convex surface which are like root hairs in form and in their manner of adherence to the soil; there is no part of this structure with any suggestion of leaflike organization or anatomy.

(2) The shieldlike structure is an axis of the first order with the root as a tab at the suspensor end, and with an axis of the second order developed laterally producing a stem apex with leaf primordia (Hofmeister (10)). In this interpretation the location of the cotyledon becomes obscure. It might be taken to be the end of the shieldlike structure away from the suspensor attachment, or it might be considered as the first leaflike structure borne on the lateral axis. As noted in (1) above, the swollen shield-shaped object does not show the structure one might anticipate in the cotyledon. The alternative, that the first leaflike structure on the lateral axis is a cotyledon, seems more reasonable since this structure has the origin and basic structure of a leaf.

(3) The third interpretation differs from the second only in designating the swollen, starchy, shield-shaped structure as the basal part of the hypocotyl rather than as an "axis of the first order", and in designating the elongate stemlike structure extending between it and the base of the cotyledon as the upper part of the hypocotyl rather than as "an axis of the second order".

Rosenberg (18) used the form of the fully developed embryo as evidence for the third interpretation, and compared it with the similar but less extreme embryo of *Halophila*. The patterns of development that have been described here support this interpretation. (*a*) The radicle never develops. (*b*) The procambial strand of the embryo develops from the 'radicle-end' towards the

center of the shieldlike part and extends at a right angle from it into the lateral protuberance. The part of the shieldlike structure beyond this emergent portion shows no differentiation of vascular tissue or any other internal or external form suggesting a leaf. (*c*) Hairs, similar to root hairs, develop all over the epidermis of the convex surface after germination, not at the 'radicle-end' alone.

The first leaflike structure produced by the stem apex at the top of the hypocotyl axis is cotyledon, there being no other structure in the embryo which admits of such an interpretation. It is similar to the coleoptile of grasses in external appearance and in position, sheathing the plumule.

It has been shown that the organization of the stem growing point of the embryo takes place at an early stage and that almost immediately it assumes a form and activity characteristic of a shoot apex. This is maintained throughout the development of the embryo and would be continued again after germination.

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HISTOLOGICAL STUDY OF HOST–PARASITE RELATIONSHIPS BETWEEN SEPTORIA GLYCINES HEMMI AND SOYBEAN LEAVES AND PODS[1]

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Abstract

Histological studies of the host–parasite relationships between *Septoria glycines* Hemmi and soybean have revealed that penetration is stomatal without the presence of an appressorium. Subsequent spread of the pathogen is intercellular in the mesophyll tissue and in the parenchyma sheath and phloem of the leaf veins. While the fungus does not show marked action in advance and requires to be in intimate contact with vital cells in order to progress through host tissue, its fruiting bodies are matured only in the holonecrotic region of the lesion. The fungus seems incapable of invading the plesionecrotic zone which borders the holonecrotic region; the role of this zone in limiting the pathogen to a discrete spot has been discussed.

Colonization of the pericarp and seed is primarily intercellular, although intracellular hyphae may be found in the sclerenchyma of the subhilum region of the seed. Infection of the seed may occur directly at the point of contact of the seed with the wall, or by systemic invasion via the placenta and funiculus. This study establishes conclusively that *S. glycines* is seed-borne in the soybean.

Introduction

The brown-spot disease of soybeans, caused by *Septoria glycines*, was first described by Hemmi (3) in Japan in 1915. Since that time it has been found in nearly all of the countries in which soybeans are produced as a crop. In 1926 the presence of the disease in the United States was confirmed by Wolf and Lehman (6), and in 1944 it was reported by Hildebrand and Koch (4) to be in Canada. In recent years in Ontario, brown spot has occurred with persistent regularity, and under conditions favorable for the development and spread of the causal organism, the disease has often assumed epidemic proportions.

The details of the symptomatology of the brown-spot disease have been presented by Wolf and Lehman (6); these authors also described the mode of entrance of the pathogen and its initial progress in the host. They suggested, too, that since the pods were attacked, the disease possibly was seed-borne. They were not able, however, to demonstrate the presence of the fungus within the tissues of the seed.

The present investigation has been concerned primarily with the pathway of infection of the soybean host. Details of penetration and colonization of the tissues of the leaf, pod, and seed are presented, together with a brief discussion of factors which possibly regulate the development of the discrete-lesion type of host response.

Materials and Methods

The susceptible soybean variety Mandarin was used throughout this investigation. All plants were grown in 4-in. pots and inoculated when the

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first trifoliate leaves were fully expanded, or when the pods were approximately half-developed. Following inoculation the plants were incubated at 22° C. in a saturated atmosphere until such time as it was established that infection had taken place. Materials for histological study were collected at 10-hour intervals subsequent to inoculation and fixed in acetic–alcohol (equal parts of 95% ethyl alcohol and glacial acetic acid), or in F.P.A. (10 parts formalin, 7.5 parts propionic acid, and 82.5 parts 50% ethyl alcohol).

Examinations were carried out either on fresh, whole mounts and razor-sections, or on fixed and embedded materials sectioned with a microtome preparatory to staining.

A culture of *Septoria glycines* Hemmi originally isolated from the soybean variety Capital was used for infection studies. Inoculum was prepared by collecting spores from a 10-day-old, vigorously sporulating Petri plate culture by flooding with approximately 100 ml. of water and rinsing several times; the resultant spore suspension was applied to the test plants with a small hand sprayer.

Results

Leaf Material

Studies on the penetration phase of infection were made from leaf segments mounted flat in lactophenol–cottonblue on a microscope slide; this procedure stained the mycelium and made it possible to observe very readily the initial progress of the pathogen and reaction of the host.

Under the experimental conditions described above, that is, a saturated atmosphere and a temperature of 22° C., penetration of the leaves of 15-day-old plants was effected within 24 hours of inoculation. Germination of the spores occurred within the first 15 hours of incubation, and there was little delay before the germ tube entered the host. Penetration was stomatal without any evidence of an appressorium, and occurred exclusively on the undersurface of the leaf, although there are stomata on both surfaces of the soybean leaf. In the substomatal chamber the penetrating hypha branched and produced a thickened runner-hypha which spread into the adjacent area and colonized subepidermally. Subsequently, this runner-hypha branched freely and ramified intercellularly throughout the spongy mesophyll. At this stage, contact between host cells and the colonizing mycelium was intimate; this resulted in a reaction which first was manifested by plasmolysis of the host cells followed by the complete disintegration of the cell contents, all within 48 hours of penetration of the stoma. Within 72 hours the pathogen had progressed upward from the spongy mesophyll and established itself in the palisade layer. Hyphal development in this latter region was considerably more extensive than that in the spongy mesophyll. Frequently there was an invasion of the bundle sheath and phloem of the veins of the leaf, but in no instance was the fungus observed to enter the xylem elements.

The above procedure, while permitting a study of the undisturbed spatial relationships of host and parasite, provided very little information regarding

their interaction; accordingly, both razor-sections of fresh material and microtome-sections of embedded material were employed. In the former instance, the material was cleared in acetic–alcohol (one part glacial acetic acid and one part 95% ethyl alcohol) and mounted in lactophenol–cottonblue. This procedure provided a very rapid method of examining material and gave a satisfactory, though temporary, mount. For permanent mounts, the material was fixed in F.P.A., embedded in Fisher's Tissuemat, sectioned at 10 μ, then stained by Johansen's safranin – fast green schedule, differentiated with orange G in clove oil, and mounted in Dee Pee X.

A study of leaf-spot lesions in cross section revealed that the contents of the host cells rapidly disintegrated, followed by the complete collapse of the walls to produce a holonecrotic area in the central portion of the invaded area. This necrosis progressed through the leaf tissues from the lower epidermis, where penetration had taken place, to the upper epidermis. For the most part, cells in the region surrounding the necrotic area remained unchanged in appearance until contacted by progressing hyphae, at which time the chloroplasts disintegrated and the cellular contents became granular. However, this situation was not maintained for long, since with the initiation of pycnidial rudiments in the holonecrotic area, there began to be an effect beyond the limits of the advancing hyphae, and the marginal cells became yellow and granular. Thus, there was developed a plesionecrotic region (2) which appeared in response to the activity of the pathogen but which never became colonized. The presence of this zone, corresponding as it did with the development of pycnidia in the holonecrotic region, seemed to serve as a barrier which limited further spread of the fungus. Such a barrier might account for the discrete nature of the *Septoria* spots, especially since there was no indication of meristematic activity in the invaded tissue, and no cicatrice was formed.

Pod Material

The histology of pod infection was studied both from fresh, razor-sections mounted in lactophenol–cottonblue, and from paraffin sections stained after the Johansen technique described above for leaf material.

Penetration of the pods was found to be stomatal and colonization of the tissue intercellular. Once the mycelium had penetrated the outer wall of the pod it spread very rapidly in the mesocarp. The cells of this latter tissue collapsed very quickly to form, along with the hyphal strands, a pseudostroma in which pycnidia were formed. Developing pycnidia often broke through into the cavity of the pod. Frequently the hyphae ramified throughout the endocarp tissue and occasionally formed a mat on the inner surface of the pod wall. In areas where the seed was in contact with the endocarp, the fungus entered the seed coat directly and formed numerous, sclerotial bodies on the seed. Colonization of the pod wall induced the collapse of the individual cells and resulted in a sunken, necrotic area which was readily observable in transverse sections of the lesion.

In addition to the direct infection of the seed at its point of contact with the pod wall, the fungus was also observed to invade the seed via the placenta

and funiculus. Abundant colonization of the pericarp in the region of the placenta usually resulted in a disruption of the vascular elements of the wall tissue, followed by an invasion of the sclerenchyma in the placental tissue. Ultimately the funiculus became colonized, and, with the formation of sclerotial bodies in the hilum area, the seed was literally separated from its attachment tissue. Hyphae which had progressed into the cells of the hilum continued to spread and occasionally formed macroscopic lesions on the cotyledons.

The presence of numerous large fruiting bodies in the colonized tissues of the placenta and funiculus suggested the possibility of seed infection from spores which might be exuded into the cavity of the pod. However, for the most part, seed invasion was as described above, that is, via the systemic pathway of the placental and funicular tissues.

Discussion

Just why many necrogenic fungi parasitic upon the leaves of plants should make very limited growth resulting in the production of discrete lesions or spots has not been satisfactorily explained. Cunningham (2), in his extensive study of the histologic changes induced by leaf-spotting fungi, found that there were two main types of host response. One of these characteristically involved the revival of a meristematic condition in certain of the leaf cells, resulting in the formation of new cells, with the ultimate production of a definite cicatrice separating the diseased portion from the healthy. The other type of response, which occurred in the large majority of cases, involved no attempt on the part of the host to lay down any tissue barrier, that is, there was no cicatrice separating the lesion from the healthy tissues. Accordingly, Cunningham concluded that "whatever may be the factor or factors governing the limitation of the necrosis caused by fungi in the leaf spot diseases of plants, it is by no means due in all cases to the formation of a cicatrice or healing tissue within the suscept, which limits the advance of the pathogene".

It has already been suggested from studies on the pycnidiaceous leaf-spotting fungi *Septoria lycopersici* (5) and *Ascochyta pisi* (1) that production of the discrete lesion is mainly a function of the developmental rhythm of the fungus, and that upon the formation of pycnidia and spores, the vital content of a colony within any one infection locus flows to the site of reproduction and is there expended. As a consequence, vegetative development of the mycelium ceases and the focus of infection becomes localized as a leaf spot.

But, in the light of the present study with *S. glycines*, also a pycnidiaceous leaf-spotting parasite, an alternate interpretation of this phenomenon may be proposed. *S. glycines* does not exhibit appreciable action in advance in its soybean host; rather, it progresses into host tissues only as long as it remains in intimate association with living cells. However, upon the formation of fruiting bodies in the holonecrotic zone, there appears evidence of changes in host cells beyond the colonized area. Presumably there is formed at this time a diffusable, phytotoxic substance, arising from host–parasite interaction, which affects vital cells some distance from the focus of infection. Since

colonization now goes no further, the possibility arises that this zone is acting as a barrier to the fungus. Histologically, the picture is strikingly reminiscent of the behavior of *S. lycopersici* on certain resistant tomato hosts (5), in which instance there is a restriction of lesion size without any concomitant displacement of the developmental rhythm of the parasite. It is suggested, then, that the inability of *S. glycines* to negotiate the zone which has been affected by the phytotoxic substance restricts the lateral spread of the mycelium; as a consequence the fungus is confined to a local zone of action as expressed by the discrete-type of lesion.

The study of infection in the pod has shown that *S. glycines* is capable of establishing itself in the pericarp, placenta, funiculus, and seed coat. Colonization of the tissues is primarily intercellular with some intracellular colonization in the sclerenchyma of the subhilum region. This latter feature of the host–parasite relationships is especially significant in view of the general opinion which holds that the sclerenchyma in the seeds of legumes acts as a physical barrier to their systemic invasion by fungi. This study establishes conclusively that *S. glycines* is seed-borne in the soybean.

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CHROMOSOMES AND RELATIONSHIPS OF KOENIGIA ISLANDICA[1]

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Abstract

The species *Koenigia islandica* L. may be assumed to be the most hardy of all annuals, since it occurs at higher altitudes and latitudes than any other therophyte. Its area of distribution, restricted to the Arctic, though with offshoots to some temperate mountains, is practically circumpolar. In Asia, it has long been known from the Tien Shan at about 40° lat. N. In Europe, its southern limits are on the Isle of Skye at about 50° 30′ lat. N., while in North America its southernmost locality was a little north of Jasper in the Canadian Rockies until its discovery a few years ago in Colorado just south of the 40th parallel. A closely related species is known to occur in the southern parts of Tierra del Fuego. Specimens from the Faeroes, Greenland, Iceland, Scandinavia, and Colorado are morphologically and cytologically inseparable, being characterized by the tetraploid chromosome number $2n = 28$. Though the genus is usually regarded as mono- or bi-typic, (1) it is pointed out that some species, often included in *Polygonum* s.lat., really belong to *Koenigia* in a somewhat wider sense; in addition to morphological similarities, they are characterized by the basic chromosome number $x = 7$, which is rare in the family and absent in the Polygoneae s.str. (2) It is also emphasized that the division of the Polygonaceae into subfamilies has not been successful since evolutionary highly distinct types are traditionally united in the subfamily Polygonoideae. (3) It is proposed that this subfamily be divided into two large subfamilies at the same time as *Koenigia* and its closest relatives be treated as a separate taxon at a very high level.

Among the very few annual plants of the high-arctic regions is a tiny member of the Polygonaceae which was described by Linnaeus (26) as the then monotypic genus *Koenigia* from Iceland. A few years earlier, the Danish botanist König had travelled in this subarctic country, and among the many remarkable plants in his collections was this most delicate of all northern therophytes. Later the species *K. islandica* was found to belong to the group of circumpolar arctic–subarctic plants with isolated localities in the central Asiatic mountains. In the subarctic regions of southern Iceland and Greenland it prefers alpine localities, while in the far north of Iceland and Greenland it is a plant of wet places in the lowland, preferably near the coast (2).

Though the genus has long been regarded as monotypic and without major variations (20, 27), some botanists have described species and varieties from different regions. The var. *ramosa* Baenitz is a modification with inordinately numerous branches mainly found in sheltered southern localities. Although it is regarded as synonymous with *K. islandica* in the Kew Index, the taxon *K. monandra* Duchaisne (in 22) hardly belongs to this genus if it is perennial as reported by its author and Meisner (39). *K. nepalensis* Don (7) has sometimes been regarded as a taxon of the collective genus *Polygonum* (cf. 39) although perhaps more correctly placed in *Koenigia* (though not in its typical

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section). The same seems to apply to *K. pilosa* Maximovicz (37), a species from the Asiatic mountains north of the Himalayas. The species *K. fertilis* Maximovicz (37) from the same regions is very closely related to the typical taxon, more than a *forma* of it as suggested by Samuelsson (47). Its main differences from the arctic taxon are in the hairiness and habit of the plant. The var. *arctica* Hadač of the typical species was described from Spitzbergen, Novaya Zemlya, and arctic Siberia (13), and it differs from the typical race mainly in the size of the floral elements and seeds, while *K. fuegina* Dusén (8) from the southernmost parts of the Tierra del Fuego differs from the northern species mainly in habit characters.

The first described and most widely spread species of the genus, *K. islandica*, grows on mudflats and gravelly, wet places in the arctic and some subarctic regions (cf. 53), where it sometimes may be so dominant as to form red patches visible from afar despite the small size of the individual plants. Though in favorable localities in Iceland, Greenland, and Scandinavia the plant may grow to 5 or even 10 cm. tall (var. *ramosa*), its average height is usually about 1–2 cm. In unfavorable habitats the minute individuals may consist of a very short stem with persistent and assimilating cotyledons, a small stem leaf, and a one-flowered inflorescence. On taller specimens, however, the stem leaf supports a false dichasium, with a group of several leaves at the apex protecting an inflorescence consisting of but a few closely packed, minute flowers which develop into small and biconvex achenes a few weeks after the germination of the original seed. In the cool and very short summers of northern Greenland and the mountains of northwestern Iceland, *Koenigia islandica* completes its development from seed to seed in less than 6 weeks.

As stated above, *Koenigia islandica* occurs as far south as the mountains of central Asia in Tien Shan at about the 40th latitude, according to Losina-Losinskaja (27). In Europe, however, the species grows in the mountains of Scandinavia south to southern Norway (21, 25) and reaches its southernmost limits in the Atlantic regions in the recently discovered locality on the Isle of Skye in the Hebrides west of Scotland at approximately 50° 30′ lat. N. (4, 44). According to Polunin (41), it is found almost everywhere in the Canadian Eastern Arctic, and southwards may reach the 56th parallel in Labrador (20), while it has not yet been reported from the Canadian Western Arctic (43) and appears to be very rare in the Yukon (42). It is known from the coastal regions of Alaska (20), while a single collection of it from the Smoky River region a little north of Jasper in the Rocky Mountains of central Alberta at about 54° lat. N. is its most southern locality so far reported from Canada. It seems to have practically no northern limits, since it grows in northern Greenland (3, 18) and northern Spitzbergen at 80° lat. N. (48, 59) although it has not been collected in Franz Joseph Land (16) and is likewise unknown from the most high-arctic regions of America (41). It is certainly the only annual plant reaching the highest altitudes and latitudes.

A great extension in the American range of this arctic therophyte was reported by Weber (58), who, together with Eilif Dahl and C. Wasser, collected

Koenigia "on gravelly polygons and sand bars of inlet streamlets feeding Summit Lake, Mount Evan, 12,700 ft. alt." on July 31, 1953. This locality is situated in the northern part of central Colorado south of the 40th parallel. It is the southernmost locality hitherto reported from North America, and at about the same latitude as the most southern stations in Asia.

Because of this tremendous range extension, it is of a great interest to study the Colorado population cytologically, in case changes in chromosome number might have become established since its isolation from the main far-north area of distribution of the species. The present writers, therefore, obtained fresh seed from material collected by Dr. W. A. Weber in September 1956, and germinated them in Petri dishes in Montreal. The seeds germinated within a few days and the plants looked like the typical highland specimens from Iceland. The chromosome number was determined in excised root tips after treatment with 0.2% colchicine for 4 hours at room temperature, and after staining and squashing in orcein. The number was determined to be $2n = 28$ and the chromosomes were small with mostly median centromere.

The chromosome number of *Koenigia islandica* was first determined as $2n = 28$ on material from West Greenland at about 65° lat. N. and from the Faeroes by Hagerup (14). That number was later confirmed on specimens from eastern Greenland by Sörensen and Westergaard (in 32) and from Jörgen Brönlunds Fjord in northern Greenland by Holmen (18) as well as from several Icelandic localities by Löve and Löve (35). Scandinavian plants were found to have $2n = 26$–28 by Edman (9) while an exactly tetraploid number, $2n = 28$, was counted by Löve (unpubl.) on Norwegian material furnished by Professor G. Edman of Stockholm. Thus, fairly many populations from the Atlantic area are cytologically known, while no reports on the cytology of Asiatic or South American material have so far been published.

It should be mentioned in this connection that several arctic species, other than *Koenigia islandica*, were reported from the Colorado Rockies by Weber (58). The most remarkable of these are perhaps *Carex capitata* L. ssp. *arctogena* (H. Sm.) Böcher, *Juncus biglumis* L., *Eutrema Penlandii* Rollins (a close relative of the arctic *E. Edwardsii* R. Br.), *Parnassia Kotzebuei* Cham., *Saxifraga foliolosa* R. Br., *Saxifraga rivularis* L. (perhaps this is rather the arctic diploid *S. hyperborea* R. Br.?), *Primula egalikensis* Wormskj., *Artemisia borealis* Pallas, and *Erigeron humilis* Grah. Certainly, it would be worth while to undertake a complete cytological study of the apparently relic flora of the high-alpine localities in Colorado not only to ascertain the identities of the arctic and Colorado species but also to check the frequency of polyploids within this peculiar flora. According to a variation of the Hagerup (15) hypothesis, mainly supported by Russian observations from Pamir, Altai, and Caucasus (49, 50, 51), such floras may be expected to be highly polyploid. The floras of the arctic regions, which apparently are closely related to this high-alpine flora, are also known to be characterized by frequencies of polyploids between 70% and 85% (cf. 11, 18, 30, 33, 35, 52, and Löve and Löve, unpubl.).

Although the tetraploid chromosome number has been found to be characteristic of *Koenigia islandica* where it is represented by its typical race in Europe and North America, numerical variations may well occur in other taxa of the genus. Judging from the stability of basic numbers in the higher taxa of the Polygonaceae (cf. 23, 28, 29, 30, 31, 34, 36), the basic number $x = 7$ will, however, certainly be found to be also typical of these taxa. But since this number is rare within the family, it is of a certain interest as an additional character for the classification of the genus within the major taxa of the family.

On morphological grounds only, several authors have made attempts to delimit the genus *Koenigia* differently from what has been generally accepted, and classify it in groups with other genera of the family. Thus, Hooker (19) regarded the genus as only a section of *Polygonum* in the broad sense. Samuelsson (47), who regarded Hooker's section *Eleutherosperma* of *Polygonum* to be so closely related to *Koenigia* that they could be grouped into the same section, endorsed this view, with the reservation, nevertheless, that Hooker's inclusion of both as sections of *Polygonum* "was right . . . . at least if the other sections are maintained under *Polygonum*." Hedberg (17), however, retained *Koenigia* as a separate genus, but proposed the inclusion of some species of *Polygonum* in the group, without, however, making the necessary transfers. While the former procedure was based on more or less superficial morphological resemblances of the heterogenous sections of a very collective genus *Polygonum*, the latter proposal rested upon pollen morphology in addition to general morphology and the then available cytological evidence, and resulted in a rather extensive division of *Polygonum* into smaller genera while at the same time enlarging *Koenigia*. As demonstrated by Löve and Löve (34), this division of *Polygonum* s.lat. is in line with cytological observations. The same can now be stated for Hedberg's (17) treatment of *Koenigia*. The basic number $x = 7$ is not encountered in any of the genera separated from *Polygonum* s.lat. though it is typical of the species of the section *Eleutherosperma* Hook. f., from the Chinese mountains and the slopes of the Himalayas, namely *P. cyanandrum* Diels, *P. delicatum* Meisn., *P. Forrestii* Diels, *P. Huberti* Lingelsh., and *P. nummularifolium* Meisn. (Löve, unpubl.). As pointed out by Hedberg (17), the pollen of these species and *Koenigia* is of the same type, their achenes are biconvex or trigonous with rather obtuse angles, the styles are very short and free, with capitate stigmas, and the branching is pseudodichotomous. Also, they are all strictly annual. The common basic number and the small size and similar morphology of the chromosomes are important additional characters of great significance for the inclusion of all these species in *Koenigia* rather than in any of the genera split out of *Polygonum* s.lat. Also, they strongly support the view that *Koenigia* in this broadened sense is a genus very well separated from all these genera in good standing. In this widened sense, *Koenigia* includes the small section *Koenigia* and the somewhat larger section *Eleutherosperma*. The nomenclatural transfers needed in this connection must, however, wait until the completion of further studies of the relationships and status of several Himalayan and Chinese taxa of the latter section.

The classification of *Koenigia* in relation to other groups of the Polygonaceae has been a matter of dispute in the past. The first monographer of the family, Meisner (38, 39), divided the family into four "subordines" or subfamilies: Eriogoneae, Polygoneae, Brunnichieae, and Symmerieae, mainly on the basis of floral and embryonal characters supported by differences in the development of the ochrea. The Polygoneae, only, was further subdivided into two tribes, the Pterygocarpeae, with the subtribes Calligoneae and Rhabarbareae, and the Apterocarpeae, with the five subtribes Ceratogoneae, Rumiceae, Eupolygoneae, Coccolobeae, and Triplarideae. The genus *Koenigia* was listed as the first of the three genera of Eupolygoneae, the other two being the collective genus *Polygonum* and *Fagopyrum*.

Bentham and Hooker (1) and also Dammer (6) made several drastic changes in the system by Meisner (38, 39) for the Polygonaceae. *Koenigia* was moved from the subfamily Polygonoideae to that of the Rumicoideae as a subtribe of the Eriogoneae in which, likewise, the western American genus *Pterostegia* and four other annuals from the southwest American mountains were also placed. This classification was again challenged by Gross (12), who divided the family into three subfamilies, or the Eriogonoideae, Polygonoideae, and Coccoloboideae. The second subfamily was divided into two tribes, the Rumiceae and Polygoneae, and *Koenigia* was placed in the latter, while the genus *Pterostegia* and the other American annuals were retained in the Eriogonoideae.

When Dammer (6) classified *Koenigia* in the Eriogonoideae close to *Pterostegia*, this was based on the opinion that the trimerous flowers with only three stamens as found in *Koenigia* must be the most primitive kind of flower in the family, while the flower with two whorls of stamens in *Pterostegia* was regarded as a derived though primitive type. This opinion was based on the hypothesis by Eichler (10) on the evolution of the floral diagrams of the Polygonaceae. It was shared by Hagerup (14), who also felt that all other floral diagrams of the family could be derived from the most simple one found in *Koenigia*. The primitiveness of *Koenigia* was doubted by Jaretzky (23) on the basis of chemical and cytological evidences, and by Edman (9) from the results of embryological investigations. The latter author moreover has also shown that the genus is clearly embryologically distinct from *Pterostegia* and *Polygonum* s.lat., with the two latter taxa more closely related in this respect. This seems to be in accord also with the anatomical studies by Laubengayer (24) and Vautier (57). The correctness of the hypothesis of the primitive nature of *Koenigia* may perhaps also be doubted in the light of the hypothesis by Mölholm-Hansen (40) that the therophytic types are the most recent of all life forms. At least within *Rumex* the annual species are highly derived (29, 45, 46).

Cytological data are of little value in the division of the Polygonaceae into primitive and advanced types as long as the original basic number and the evolution of chromosome morphology within the family remain unknown. Combined with other characters, however, they are of great assistance in

grouping related species and genera and in separating those which cannot be regarded as closely connected to each other from the point of view of evolution. It is true that the basic number of *Pterostegia* and the other annual western North American genera grouped with *Koenigia* by Dammer (6) is $x = 7$ (54, 55, and Löve, unpubl.) as in *Koenigia*, so that this fact alone does not contradict this classification. The morphology and size of the chromosomes, however, is so fundamentally different in these genera (Löve, unpubl.) that there can be no doubt that they are not closer related to each other than they are to other genera within the family characterized by the same basic number, as, e.g. *Oxyria* (cf. 56), *Acetosella* (cf. 28), or *Ruprechtia* (5). As far as can be seen from the available data, the division of the family Polygonaceae into three subfamilies is insufficient from the cytological as well as morphological and anatomical points of view, so that although the classification by Gross (12) of the Eriogonoideae and Coccoloboideae is likely fairly natural, the groups he classified as tribes within the Polygonoideae are much more heterogenous. Cytological studies have yielded extensive evidence supporting the view that not only are the tribes of this subfamily so remotely related that each can claim the status of subfamily with the same strength as the above groups, but also the genus *Koenigia* and its closest relatives within the Polygoneae have most probably reached such a distinction that their classification in a separate subfamily seems justified.

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FACTORS AFFECTING OXIDATION AND PHOSPHORYLATION BY SOYBEAN MITOCHONDRIA[1]

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Abstract

Active particles, presumed to be mitochondria, have been isolated by differential centrifugation from the hypocotyls of etiolated soybean seedlings. These particles were capable of oxidizing all Krebs cycle intermediates tested, although fumarate and oxaloacetate were oxidized relatively slowly. Oxygen uptake in the presence of succinate, pyruvate, or ketoglutarate was increased two to four times by the addition of ATP, $MgSO_4$, and cytochrome *c*. Maximum activity was obtained when both sucrose and phosphate were added to the grinding and suspending media, and when the reaction mixture was buffered near pH 7.0. Endogenous oxidation was reduced 50% by one washing and at the same time the oxidation of exogenous substrate was increased two to three times. Average P:O ratios of 0.4, 0.8, and 0.9 were obtained without added hexokinase or fluoride for succinate, pyruvate, and ketoglutarate, respectively. These ratios were increased to 0.8, 1.2, and 1.5 by the addition of hexokinase. In all cases, 10^{-2} *M* NaF decreased oxygen uptake 30 to 40% without affecting P : O ratios. Isolated soybean particles appear to be similar in most respects to particles from other plants. Certain differences were found and are discussed.

Introduction

In recent years evidence has accumulated that the enzymes involved in aerobic plant respiration and phosphorylation are located in discrete cytoplasmic particles called mitochondria (2, 3, 7, 8, 10–15, 17, 22). The cytology of plant mitochondria has been reviewed by Newcomer (19), and the biochemical activities by Millerd and Bonner (16). Particles considered to be mitochondria have been isolated from several species and their biochemical activities studied. Included in these investigations have been particles from seedlings of mung beans (2, 15, 17), white lupines (3, 5, 25), peas (4), oats (20, 23), barley (25), kidney beans (1), and from cauliflower buds (11, 12, 13), avocado fruits (18), and the spadix of *Arum maculatum* (9).

This study of conditions of particle isolation and oxidative and phosphorylative activity of particles from etiolated seedlings of the soybean was carried out in connection with an investigation of the effects of herbicides on particle activity to be published elsewhere.

Materials and Methods

Procedures followed in this investigation were based on those of Millerd *et al.* (17) and Laties (11, 12, 13). Soybeans (*Glycine max* (L.) Merr. var. Hawkeye) were surface sterilized for 10 minutes in 0.5% NaOCl and soaked in tapwater for approximately 24 hours. They were then planted in sterilized sand in shallow enamel pans and grown in the dark in 26° C. incubators for 4 days.

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At this time, the hypocotyls which were about 10 centimeters in length were harvested and transferred to a beaker in an ice bath. Wherever possible in the procedure, the plant materials were kept close to 0° C.

The 80 to 100 g. of hypocotyls used in each experiment was divided into two equal lots, and each was macerated for approximately 2 minutes in a chilled mortar with cold quartz sand and 25 ml. of sucrose–phosphate solution. Except where otherwise noted, this grinding solution contained 0.4 *M* sucrose and 0.1 *M* phosphate ($KH_2PO_4+Na_2HPO_4$) and had a pH of 7.1. After grinding, the pH of the brei was approximately 6.8. The brei was strained through cheesecloth to remove most of the sand and tissue debris and centrifuged for 5 minutes at $500\times g$ in a refrigerated centrifuge. The supernatant was decanted into four plastic tubes and recentrifuged at $10{,}000\times g$ for 15 minutes. The supernatant was then discarded, and the residue in each tube was resuspended in 2 ml. of cold solution, 0.4 *M* in sucrose and 0.1 *M* in pH 6.9 phosphate (except where otherwise noted). The total volume was brought to 20 ml. and this suspension recentrifuged in two tubes at $10{,}000\times g$ for 15 minutes. The sediment from this second high-speed centrifugation was homogenized in a glass tube by means of a power-driven plastic pestle with 8 or 15 ml. of sugar–phosphate solution. Either 0.5 or 1.0 ml. of this homogenate containing approximately 0.4 mg. of nitrogen (micro-Kjeldahl) was used in each Warburg flask.

Oxygen uptake and carbon dioxide evaluation were determined manometrically at 30° C. in air by the method of Warburg as outlined by Umbreit *et al.* (24). Carbon dioxide was measured at the end of experiments, mainly in the same two-side-arm flasks used for oxygen uptake. Manometric readings were generally taken at 5 minute intervals for a 30 minute period. During this period there was some decrease in rate, as has been observed with other plant particles, but the gas exchange under these conditions was proportional to particle concentration up to 0.6 mg. of particle nitrogen per flask. Gas exchanges are reported as net values (total minus endogenous) and are expressed as microliters per mg. nitrogen per hour ($Q_{O_2}(N)$). Since there was close agreement between replicate flasks in a given experiment (e.g., a mean difference of 4.3% with a standard deviation of 3.7% with succinate as substrate) only one flask per treatment was used in each experiment. Experiments were then repeated at least three times.

In experiments in which phosphorylation was measured, inorganic phosphate was determined at zero time and at the end of 35 minutes by the method of Fiske and Subbarow (6). The difference in these two determinations was considered to represent the amount of inorganic phosphate esterified by the enzyme system (2).

The following cofactors were used: adenosine-5-phosphate (AMP) and adenosine diphosphate (ADP) (chromatographically pure, Pabst Laboratories); adenosine triphosphate (ATP) (chromatographically pure, Schwarz Laboratories); coenzyme A (75% pure, equivalent to 300 Lipmann units per g., Pabst Laboratories); cytochrome *c* (90–100% pure, Sigma Chemical Co.);

diphosphopyridine nucleotide (DPN) (chromatographically pure, Schwarz Laboratories); and hexokinase (contained 28,000 Kunitz-McDonald units per g., Pabst Laboratories).

Experimental Results

Oxidation of Krebs Cycle Intermediates

All of the Krebs cycle acids tested were oxidized in the presence of isolated soybean particles, although some much slower than others. The largest oxygen uptake was observed with succinate as substrate (Table I), followed by pyruvate, citrate, and ketoglutarate. Without the addition of malate, pyruvate oxidation was negligible and all future references to pyruvate oxidation will be to the pyruvate–malate mixture. The quantity of malate used as "sparker" was sufficient to bring about a small amount of oxidation by itself. Glutamate also was utilized indicating the ability of isolated soybean mitochondria to oxidize at least this particular amino acid. The rates of fumarate and oxaloacetate oxidation were low, although malate was utilized more rapidly. Millerd *et al.* (17) and Dow (5) also found that fumarate oxidation was slower than the oxidation of other intermediates by mung bean and lupine mitochondria.

TABLE I

ABILITY OF SOYBEAN MITOCHONDRIA TO OXIDIZE VARIOUS KREBS CYCLE INTERMEDIATES*

Substrate	$Q_{O_2}(N)$	$Q_{CO_2}(N)$	R.Q.	
			Theoretical†	Observed
Pyruvate	7	—	—	—
Malate (0.0017 *M*)	25	—	—	—
Pyruvate‡	168	200	1.20	1.2
Citrate	130	205	1.33	1.6
Ketoglutarate	123	169	1.25	1.4
Glutamate	60	70	1.11	1.2
Succinate	319	96	1.14	0.3
Fumarate	13	11	—	—
Malate	65	75	1.33	1.2
Oxaloacetate	17	43	—	—

*All flasks contained substrate, 0.02 *M*; ATP, 0.0005 *M*; $MgSO_4$, 0.001 *M*; sucrose, 0.2 *M*; pH 6.9 phosphate, 0.05 *M*.
†Theoretical values based on complete oxidation to carbon dioxide and water.
‡Malate, 0.0017 *M*, added.

The R.Q. data in Table I are some indication of the type of reactions. Except for succinate the observed values are close to those expected for complete oxidation, but in themselves, of course, do not prove complete cycle operation. The very low value for succinate would be expected from the slow rate of fumarate oxidation. Average R.Q. values for pyruvate, ketoglutarate, and succinate obtained with soybean mitochondria were in agreement with those reported for pyruvate with mung bean mitochondria by Millerd *et al.* (17) and for ketoglutarate and succinate with *Arum* particles by Hackett and Simon (9).

Effect of Procedure on Oxidative Activity

Composition of Grinding and Suspending Media

The tonicity, phosphate content, and pH of solutions in which plant mitochondria are suspended have been shown to influence the oxidative activity of these particles (13, 15, 17). The results of experiments to test the effects of these factors on soybean mitochondria are presented in Table II, in which averages of two complete experiments are given. In the first experiment of this type (A, Table II) soybean hypocotyls were ground in the mortar either with a solution of 0.4 *M* sucrose and 0.05 *M* KCl, or with a solution of 0.4 *M* sucrose and 0.05 *M* tris (trishydroxymethylaminomethane) buffer. Part of each pellet centrifuged down by the first high-speed spinning was resuspended (washed) in an aliquot of the original sucrose–KCl solution and part in a 0.4 *M* sucrose–0.05 *M* phosphate solution. After recentrifugation and resuspension in a solution of the same composition as the washing solution, each of the four homogenates thus obtained was tested for ability to oxidize succinate, ketoglutarate, and pyruvate.

TABLE II

EFFECTS OF COMPOSITION OF GRINDING AND SUSPENDING MEDIUM ON OXYGEN UPTAKE BY SOYBEAN MITOCHONDRIA*

Substrate	Grinding medium	Total molarity	pH after grinding	Washing and suspending medium	$Q_{O_2}(N)$
		A experiments			
Succinate	S+KCl	0.45	6.4	S+KCl	218
"	S+KCl	0.45	6.4	S+PO_4	352
"	S+tris	0.45	6.9	S+KCl	221
"	S+tris	0.45	6.9	S+PO_4	438
Ketoglutarate	S+KCl	0.45	6.4	S+KCl	0
"	S+KCl	0.45	6.4	S+PO_4	42
"	S+tris	0.45	6.9	S+KCl	114
"	S+tris	0.45	6.9	S+PO_4	175
Pyruvate	S+KCl	0.45	6.4	S+KCl	23
"	S+tris	0.45	6.9	S+KCl	206
"	S+tris	0.45	6.9	S+PO_4	206
		B experiments			
Succinate	S+PO_4	0.45	6.9	S+PO_4	570
"	S+PO_4	0.45	6.9	PO_4	208
"	PO_4	0.10	6.9	S+PO_4	113
"	PO_4	0.10	6.9	PO_4	62
Ketoglutarate	S+PO_4	0.45	6.9	S+PO_4	202
"	S+PO_4	0.45	6.9	PO_4	25
"	PO_4	0.10	6.9	S+PO_4	43
"	PO_4	0.10	6.9	PO_4	0
Pyruvate	S+PO_4	0.45	6.9	S+PO_4	228
"	S+PO_4	0.45	6.9	PO_4	13
"	PO_4	0.10	6.9	S+PO_4	30
"	PO_4	0.10	6.9	PO_4	0

*All flasks contained substrate, 0.02 *M*; ATP, 0.0005 *M*; $MgSO_4$, 0.001 *M*; sucrose (S), 0.2 *M*; phosphate, 0.05 *M*; tris, 0.05 *M*. Malate, 0.0017 *M* added with pyruvate. Grinding media contained sucrose, 0.4 *M*; phosphate, 0.05 *M* or 0.1 *M*; tris, 0.05 *M*; KCl, 0.05 *M*.

With succinate as substrate, oxygen uptake was not affected by the addition of tris buffer to the grinding medium. A large increase, however, was obtained with both ketoglutarate and pyruvate. The addition of phosphate to the washing and suspending medium increased oxidation of succinate and ketoglutarate regardless of the grinding medium but had no effect on pyruvate oxidation. With all substrates the greatest oxygen uptake was obtained when the grinding medium was buffered and phosphate was added to the suspending solution. These results led to experiments in which phosphate was used in the grinding medium, with and without sucrose, as well as in the suspending solution. Data from experiments of this type are presented in the second part (B) of Table II. With all substrates, oxygen uptake was greatly reduced when either sucrose or phosphate was omitted from either the grinding or suspending solution. No ketoglutarate or pyruvate oxidation occurred when sucrose was omitted from both the grinding and suspending media, although succinate was oxidized to a slight extent. However, particles prepared by grinding in phosphate alone (0.1 *M*) were capable of some oxidation if sucrose was added to the suspending medium. Since isolation, washing, and suspension of soybean particles in hypertonic sucrose–phosphate buffered near pH 7 gave the greatest activity, this procedure was used throughout the present study unless otherwise noted.

Although maintenance of hypertonicity has been found desirable by all workers with plant mitochondria, the necessity for added phosphate does not appear to be general. Laties (11, 12, 13) and Tager (23) did not add phosphate to sucrose solutions in which active mitochondria were isolated from cauliflower buds and *Avena* coleoptiles. However, increased activity of soybean particles in the presence of added phosphate was similar to results obtained by others with mitochondria from mung beans (17), lupines (2), and kidney beans (1).

Washing

Considerable endogenous oxygen uptake remained after one washing. With succinate, it varied from 10 to 20% of that with added substrate. The effects of further washing of the particles in sucrose–phosphate are shown in the representative experiment in Table III. Although the oxidation rate with pyruvate was below average in this case, the relative activities with

TABLE III

EFFECT OF WASHING ON OXYGEN UPTAKE ($Q_{O_2}(N)$) BY SOYBEAN MITOCHONDRIA*

Substrate	Number of washings			
	0	1	2	3
Succinate	269	591	542	540
Pyruvate	35	98	68	50
None	102	48	35	27

*All flasks contained substrate, 0.02 *M*; ATP, 0.0005 *M*; $MgSO_4$, 0.001 *M*; sucrose, 0.2 *M*; pH 6.9 phosphate, 0.05 *M*. Malate 0.0017 *M*, added with pyruvate.

successive washings were typical for pyruvate oxidation. Each washing after the first decreased oxygen uptake but also decreased activity with added substrate. From such experiments the practice of a single washing was adopted.

pH of Reaction Mixture

The effect of varying the pH of the reaction mixture on oxygen uptake was investigated with succinate, pyruvate, and ketoglutarate as substrates. The pH in each flask was adjusted by appropriate additions of phosphate buffers (KH_2PO_4–Na_2HPO_4) to give a final concentration of 0.05 *M* phosphate and was measured at the end of the experiment. The data in Fig. 1 show that the optimum activity with all three substrates was in the range of pH 6.5 to pH 7.1. Based on these results, the reaction mixtures in other experiments of this study were buffered at pH 6.9. This is a little lower than that used by most other investigators. Millerd *et al.* (17, 18) worked at a pH of 7.1; Laties (13) and Tager (23) at 7.3. Soybean mitochondria appeared to be about as active at pH 7.1 as at 6.9, but showed reduced activity, especially toward succinate, at pH 7.3. Whether this sensitivity to higher pH values is a distinct property of soybean mitochondria or due merely to the isolation procedure was not determined.

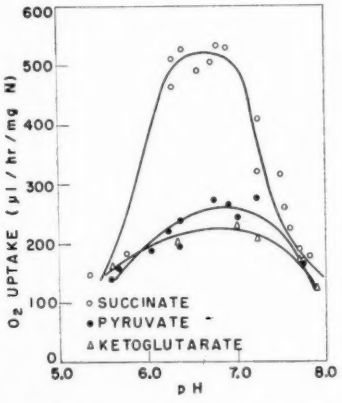

FIG. 1. Effects of pH of reaction mixture on the oxidative activity of soybean particles.

Addition of Cofactors

Data from representative experiments showing the effects of ATP and $MgSO_4$ on oxidation of succinate, pyruvate, and ketoglutarate are presented in Table IV. With all three substrates, oxygen uptake was increased by the addition of either $MgSO_4$ or ATP and still further increased if both were added. This increase was approximately additive with all three substrates. Pyruvate and ketoglutarate oxidations were stimulated more by ATP than by magnesium ions, and succinate oxidation showed the reverse. Pyruvate and succinate oxidation was further increased by the addition of cytochrome *c*.

TABLE IV

EFFECT OF ATP, $MgSO_4$, AND CYTOCHROME *c* ON OXYGEN UPTAKE ($Q_{O_2}(N)$) BY SOYBEAN MITOCHONDRIA*

Substrate	Cofactors added			
	0	Mg	ATP	Mg+ATP
Succinate	200	397	319	570
Ketoglutarate	107	141	174	202
Pyruvate	70	84	107	156

*All flasks contained substrate, 0.02 *M*; sucrose, 0.2 *M*; pH 6.9 phosphate, 0.05 *M*. Added were ATP, 0.0005 *M*; $MgSO_4$, 0.001 *M*; cytochrome *c*, 0.000028 *M*. Malate, 0.0017 *M*, added with pyruvate.

This cofactor had little effect on oxygen uptake in the presence of ketoglutarate. The effects of the cofactors DPN and coenzyme A in mixtures containing magnesium ions and ATP were also investigated. DPN and CoA alone had little effect on either succinate or pyruvate oxidation, but in conjunction with cytochrome *c* they increased oxygen uptake markedly in the presence of both substrates. In the presence of ketoglutarate, both DPN and CoA stimulated oxygen uptake, and cytochrome *c* had little additional effect. In general, oxidation of soybean mitochondria seemed to be stimulated by the addition of ATP, $MgSO_4$, DPN, CoA, and cytochrome *c*. Important exceptions appeared to be the relative insensitivity of the ketoglutarate system to cytochrome *c*, and the pyruvate system to DPN and CoA. Further work is required to characterize fully the response of soybean particles to cofactors, especially DPN, CoA, and cytochrome *c*.

In studies on phosphorylation it was desirable to replace ATP with AMP as a source of adenylate. Experiments were therefore set up to verify the equivalence of AMP, ADP, and ATP in stimulating oxygen uptake by soybean mitochondria. The results of a typical experiment of this type are shown in Table V. It may be seen that the oxidation of succinate, pyruvate, and ketoglutarate was stimulated to approximately the same extent by the addition of each of these adenylates. Similar findings have been reported by Millerd *et al.* (17) for pyruvate oxidation by mung bean particles.

TABLE V

EQUIVALENCE OF ATP, ADP, AND AMP IN THE STIMULATION OF OXYGEN UPTAKE ($Q_{O_2}(N)$) BY SOYBEAN MITOCHONDRIA*

Substrate	Adenylate added			
	0	ATP	ADP	AMP
Succinate	308	445	438	433
Ketoglutarate	141	208	213	220
Pyruvate	170	220	217	213

*All flasks contained $MgSO_4$, 0.001 *M*; sucrose, 0.2 *M*; pH 6.9 phosphate, 0.05 *M*. Malate, 0.0017 *M*, added with pyruvate.

Phosphorylation Ability

Mitochondria isolated from other plants have been shown to utilize part of the energy of respiration in the formation of high-energy phosphate bonds (2, 12, 15, 17). Therefore, experiments were set up to investigate the disappearance of inorganic phosphorus from the reaction mixture during the oxidation of certain substrates by soybean mitochondria. It was assumed that each micromole of inorganic phosphate that disappeared was attached through a high-energy bond to a micromole of adenylate, as has been shown with mung bean mitochondria (2, 15).

Particles were prepared in the usual manner except that a lower concentration of phosphate was used in the reaction mixture (6.25×10^{-3} *M*). At this concentration oxygen uptake was considerably reduced (220 μl./hour as compared with 400 μl./hour at 0.05 *M* phosphate). Other details of the procedure have been outlined in the methods section. AMP and glucose were added to all flasks except where otherwise noted, AMP as an acceptor for phosphate, as suggested by Bonner and Millerd (2), and glucose to ensure the trapping of high-energy phosphate as glucose-6-phosphate. This trapping reaction is mediated by the enzyme hexokinase, which has been shown to be associated with the mitochondria of potato tubers (21) and of mung beans (2). If it is similarly associated with soybean mitochondria in sufficient quantity to transfer all high-energy phosphate formed to glucose-6-phosphate, no increase in the rate of disappearance of inorganic phosphate would be expected from the addition of exogenous hexokinase. That such was not the case is shown in Table VI, which also shows the effects of added NaF on oxygen uptake and phosphate disappearance.

Some phosphorylation was evident in the presence of each of the three substrates tested when neither hexokinase nor fluoride was added. P:O ratios of 0.4, 0.8, and 0.9 were obtained with succinate, pyruvate, and ketoglutarate, respectively. These ratios were raised to 0.8, 1.2, and 1.5 by the addition of 2.5 mg. hexokinase per flask. The addition of fluoride, by itself or with hexokinase, had little effect on P:O ratios. Both oxygen uptake and

TABLE VI

EFFECTS OF ADDED HEXOKINASE AND NaF ON OXIDATION AND PHOSPHORYLATION BY SOYBEAN MITOCHONDRIA*

Material added	Oxygen uptake (μatoms/hr.×flask)			Phosphate uptake (μmoles/hr.×flask)			P:O		
	S†	P†	K†	S	P	K	S	P	K
None	13.9	9.0	9.1	5.8	7.6	8.5	0.42	0.84	0.93
Hexokinase (2.5 mg./flask)	14.3	8.9	8.3	11.2	11.5	12.2	0.78	1.18	1.47
NaF (10^{-2} *M*)	9.3	6.3	6.2	—	—	6.3	—	—	1.01
Hexokinase+NaF	10.8	5.9	6.8	8.6	6.8	9.5	0.79	1.15	1.40

*All flasks contained substrate, 0.02 *M*; AMP, 0.0005 *M*; $MgSO_4$, 0.001 *M*; glucose, 0.1 *M*; sucrose, 0.1 *M*; pH 6.9 phosphate, 0.00625 *M*. Malate, 0.0017 *M*, added with pyruvate.
†S = succinate, P = pyruvate, K = ketoglutarate.

phosphate disappearance were inhibited to about the same extent by the concentration of fluoride used, 10^{-2} *M*. These results were surprising in the light of data in the literature on the effect of fluoride. Some inhibition of oxygen uptake (20%) was noted by Bonner and Millerd (2) upon the addition of 10^{-2} *M* fluoride, but the P:O ratio was increased considerably. Since it is assumed that ATP is being formed from ADP and AMP during phosphorylation, such an increase would be expected if fluoride were acting to inhibit ATPase activity, as has been demonstrated (15). The ATPase activity of soybean mitochondria was too low to release a measurable amount of inorganic phosphate from 5×10^{-4} *M* ATP in 30 minutes at 30° C. This might account for the inability of added fluoride to increase the P:O ratios of this system with the concentration of adenylate used in these experiments.

Since 10^{-2} *M* NaF was found to be a strong inhibitor of oxygen uptake by soybean mitochondria in the presence of all substrates tested, the effect of various lower concentrations was investigated. Succinate, ketoglutarate, and pyruvate oxidation were all slightly inhibited (8–10%) at concentrations of fluoride as low as 10^{-4} *M*. Effects of concentrations of fluoride lower than 10^{-2} *M* on phosphorylation and P:O ratios were not investigated.

Discussion

In general, the oxidative activity of soybean particles was similar to that of particles from other plants. The various Krebs cycle intermediates tested were all oxidized although fumarate and oxaloacetate were oxidized only slowly. Similar results have been obtained by Millerd *et al.* (17) with mung bean particles and by Dow (5) with lupine particles.

Oxygen uptake by isolated soybean particles was greatly reduced when either sucrose or phosphate was omitted from the grinding or suspending solution. Considering these results in the light of observations by others (1, 5, 11–13, 15–17), it seems apparent that isolated plant particles must be kept immersed in a slightly hypertonic solution to maintain a high level of activity. Data obtained in the present investigation in experiments in which high solute concentrations (0.5 *M* to 1.0 *M*) were added to soybean particle preparations indicated that injury might also result from too high tonicity. The optimum level seemed to be about 0.4 *M* in the grinding medium (13, 17).

Although the necessity for added sucrose or mannitol in the suspending solution has been observed by all investigators, the same is not true for phosphate. Laties (11, 12, 13) and Tager (23) did not add phosphate to the sucrose solutions in which mitochondria were isolated from cauliflower buds and *Avena* coleoptiles. The increased activity of soybean particles in the presence of added phosphate was similar to the results of Millerd (15) with mung beans; Dow (5), with white lupines; Hackett and Simon (9) with *Arum maculatum*; and Beaudreau and Remmert (1) with kidney beans. Millerd's experiments (15), indicated that the added inorganic phosphate acted to inhibit phosphorylytic breakdown of some essential substance or

substances. If this is the correct explanation, there must be a greater tendency for this breakdown to occur in particles isolated from some plants, including soybeans, than from others.

Most investigators have subjected mitochondrial suspensions to one or more washings during the isolation procedure (5, 11, 15, 23). Millerd (15) and Laties (11) reduced endogenous oxygen uptake to a negligible amount by one washing, but Dow (5) and Tager (23) were unable to do so. Similar difficulty was encountered in the present study, where endogenous oxygen uptake of once-washed particles varied from about 10 to 20% of the oxygen uptake in the presence of succinate. This endogenous activity could be reduced by further washings, but the activity of pyruvate oxidizing enzymes was also markedly reduced by more than one washing. The single washing which was used routinely, however, not only decreased endogenous oxygen uptake about 50%, but also doubled succinate oxidation and tripled pyruvate oxidation. Apparently both endogenous substrate and some interfering substance or inhibitors of succinate and pyruvate oxidation were removed. The decrease in pyruvate oxidation with more than one washing was probably due to a loss of enzymatic activity over the longer periods of time involved between initial isolation and addition to the Warburg flasks. The succinoxidase system, which did not show this decline in activity, is apparently more stable.

The pH optimum for soybean particle activity was demonstrated to be about 6.9, which is only slightly lower than that used by other investigators (11, 17, 23). In general, cofactor requirements were also similar to those for particles from mung beans (15, 17), cauliflower buds (11, 12, 13), *Avena* coleoptiles (23), and kidney beans (1), although a strong cytochrome *c* response was obtained, which differs from the results of Millerd *et al.* (17) and Tager (23). Millerd *et al.* (17) relate necessity for added cytochrome *c* to the method of preparation, since they were able to produce mung bean particles that did respond to this cofactor by omitting or lowering the concentration of phosphate in the grinding medium. Since phosphate was used in the present study and other steps in the isolation procedure were similar to those of Millerd *et al.* (17), it would seem that cytochrome *c* saturation of isolated particles is influenced by the species of plant from which the particles are taken as well as by the method of isolation.

Soybean mitochondria have been shown to take up inorganic phosphate during oxidation and presumably to esterify it into high-energy phosphate bonds, as has been observed with particles from other plants (2, 12, 15, 17). In the present study, phosphate disappearance was enhanced by the addition of hexokinase. In the presence of glucose, hexokinase mediates the transfer of high-energy phosphate from ATP to glucose-6-phosphate. A receptor molecule for phosphate esterification is thus liberated and the glucose–hexokinase acts as a trapping system for all phosphate subsequently esterified. Bonner and Millerd (2) obtained no increase in P:O ratios by adding hexokinase, and stated that mung bean hexokinase is firmly bound to the

mitochondria and does not limit the phosphorylation reaction. This apparently is not the case with soybean mitochondria. In this respect, they resemble cauliflower bud mitochondria, since Laties (11) found that the latter particles did not contain enough hexokinase to permit maximum oxidation. However, P: O ratios obtained with soybean particles (about 1.0) were closer to those obtained with mung beans (2) than to those of Laties (13) with cauliflower. It would appear that the phosphorylative enzymes of both mung beans and soybeans are more easily damaged during preparation than those of cauliflower.

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GROWTH AND MORPHOGENESIS IN THE CANADIAN FOREST SPECIES

II. SPECIFIC INCREMENTS AND THEIR RELATION TO THE QUANTITY AND ACTIVITY OF GROWTH IN PINUS RESINOSA AIT.[1]

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Abstract

Two measures are needed to describe numerically the activity of the internodal cambium in terms of annual increment. These are "specific wood volume increment", a measure of additive growth and "specific increment of cambial area", a measure of multiplicative growth. The mean area of the internodal cambium is the basis of reference for both since it is the measure of that which is active in growth. The former measure of specific growth is numerically equal to ring width and the manner of its factorial control has already been considered. Data for the latter are new.

The geometry of the apical meristem and its products is too complex for the ready computation of specific terminal growth, but it can be shown empirically that internode length is a valid measure of apical activity. Analysis of internodal wood volume growth into its three linear components leads to the conclusion that the determinants in control of wood growth act mainly through their effect upon apical activity and upon specific increment of cambial area.

I. Introduction

The growth of a tree is an integral process but it has innumerable aspects all of which must be explored if a comprehensive view of growth is ever to be achieved. In the first paper of this series (13) the authors gave an account of the control of cambial and terminal growth in *Pinus resinosa* by factors inherent in the organism itself and by factors of extraneous origin. In the present paper we propose to consider other aspects of the same data, attempting thus to increase our understanding of the integral growth of the tree by regarding from a different point of view numerical data that have already been recorded and examined in considerable detail.

Our first contribution was based upon original data for internodal ring growth in a comparatively small number of trees of a single species, but there is reason to believe that the principles we deduced and applied are of general validity. At the time our paper was published, and until quite recently, we were unaware of the previous work of Onaka (20), published in Japanese, which in certain respects more closely resembles our own than the work of any other author. In these circumstances it is perhaps fortunate that we made no attempt to discuss the bearing of our results and conclusions upon the views of others. Neither have we published any attempt to relate the results of our studies of growth in trees to growth studies in other organisms. We have deliberately withheld discussion because our method, hypotheses, and conclusions have yet to be examined in the light of additional data of our

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own that still await presentation. Also we have been aware for some time that our procedures and concepts were being put to the test in independent researches involving a far wider range of species, geographical distribution, and vicissitude than our own. The work of Mott, Nairn, and Cook (19), which we have had the privilege of reading before its publication, confirms our results and conclusions and extends the usefulness of our method. But it is safe to suppose that more work of both the extensive and the intensive kind will be needed to achieve a fully satisfactory clarification of even the technical problems involved in growth studies of this character.

In the course of our work we could not fail to be impressed by the almost incredible fact that though trees of geometrical habit such as *P. resinosa* would appear to be among the most nearly ideal of living organisms in which to examine problems of biological growth, yet, the enormous literature of tree growth has contributed little or nothing to the comparative study of growth. The reason for this would seem to be that growth data for trees, so often related to problems of forestry, have too seldom been regarded by their authors as data for biological growth and this has had inevitable effects upon their formulation. For instance, the advantages of adopting specific growth where a measure of growth activity is required appear to have pretty generally gone unremarked. Perhaps for this reason the fact that ring width is numerically equal to a certain measure of specific annual increment seems not at all widely appreciated amongst students of tree growth. In our earlier paper we adopted ring width as a measure of cambial activity (Duff and Nolan (13, p. 477)) but we deliberately postponed considering the reasons for this adoption. These problems will be resumed here.

In the immediately succeeding section of this paper we shall review first the biological considerations upon which our choice of the several measures of quantity depends. We are thus enabled to arrive at some conception of the functional significance of the proposed measures of growth. The evaluation of these quantities for the limited purpose of analyzing annual increments of internodal growth becomes then a matter of elementary geometry. In the third section the primary data for a certain typical tree are examined in terms of the growth measures derived in the second and the indications of the derived data for the action and relative importance of the determinants of growth activity are recorded. The final section extends the observations to trees grown under contrasting conditions and the comparative study helps to distinguish the conclusions of general validity from those of merely particular application.

II. Derivation of Data

The primary data of this paper have all been derived and presented in the preceding communication (13). The methods, the history of the trees, and the character of the sites upon which they grew as well as the data for ring width are already accessible and the reader is referred to that paper for all such matter. Our method of examining the variations of growth by surveying

its distribution in the tree in terms of sequences is also made clear in the paper referred to. We here propose to compute derivative data other than those which we have considered heretofore. The first such computations will be for specific growth, the interest of which originates in biological considerations rather than in considerations of forestry practice.

1. Specific Growth, Its Biological and Computational Basis

Wherever, as here, a principal object in the study of growth is to formulate the action of its controls, it is clear that it is not the amount but the activity of growth that must be considered. Accordingly, what chiefly counts is the rate of production per unit of that which is active in growth, that is to say specific growth, and the best approximation to the ideal measure of growth activity would therefore be specific growth rate. In a future paper we propose to consider specific growth rates but these require the presentation of new primary data and in the present communication we shall restrict ourselves to derivatives that can be extracted from our published primary data for annual increment and it is therefore specific annual increment that we shall perforce have to deal with. The latter is not the full equivalent of specific growth rate but the two have functional homologies sufficient to permit of an instructive advance in the analysis of annual growth in trees through the consideration of specific annual increments.

In order to compute a numerical value for any form of specific growth we must first identify and evaluate that which is active in growth. In many instances of growth in higher organisms there is ambiguity as to what the active thing is; in others we encounter formidable difficulties in arriving at a satisfactory numerical evaluation. In the tree the active agency can easily be specified. As in nearly all morphogenetic growth in plants, it is one or other of the meristems. Omitting in the interest of simplification all intercalary and other auxiliary growth functions, we have two meristems to consider in our species, the primary, apical meristem and the secondary cambium.

The numerical evaluation of the apical meristem as a center of growth is highly problematical. A comprehensive account of terminal growth must include not only the formation of the major differentiated products of the terminal shoot but also a concomitant change in the amount of apical meristematic tissue as well. This duality of the growth process must always be taken account of wherever growth is meristematic and the growth of the meristem itself as well as the formation of its differentiated products must be considered. In the following subsection we confront this problem in the relatively simple instance of the internodal cambium, which increases progressively as long as the tree is growing radially at all, but the geometrical complexity of the growth process at the apical meristem makes it necessary to postpone any attempt at analytical treatment of apical growth until more data are before us. We may simply remark at this point that the growth of the apical meristem itself is involved with problems of lateral bud formation at the apex and subsidiary bud formation along the flanks of the terminal shoot which make it improbable

that a satisfactory specific annual growth of the meristem will be readily calculable. Regarding the other component of the dual growth process, we have already shown (13) that the sequence of annual terminal growth corresponds to and may be reconciled, though not in any simple way, with third type sequences of internodal ring width, which is a measure of specific increment, as we shall see. Accordingly, on these empirical grounds and within appropriate limits we may employ internode length as a numerical measure of apical growth activity. The formal justification for regarding ring width as a proper if partial measure of cambial growth activity will be considered in the following subsection.

By contrast with the apical meristem the cambium is easily given a numerical evaluation, provided the data for annual increment are derived for each internode separately, thus permitting the evaluation to be based upon the dimensions of internodal cambia. Here, corresponding cellular and organo-differentiation processes occur contemporaneously and the growth product itself, although it also is compound rather than elementary, remains measurable because of its relatively simple geometry and convenient dimensions. We shall still further simplify by regarding the annual product as merely the internodal secondary wood ring of that year together with an increment of growth in the cambium itself.

However it may be defined histologically, the geometry of the internodal cambium is effectively that of a two-dimensional tissue system. We propose to evaluate it accordingly in terms of its area. Thus, area of the internodal cambium, Ac, or its equivalent becomes the measure of the thing that is active in radial growth and to which, therefore, we shall refer the amount of annual cambial growth product in the instances with which we shall deal in this paper.

2. Specific Annual Increment

Biological Duality

The activity of the cambium comprises two different kinds of tissue growth which have their cellular basis in divisions organized in two correspondingly different kinds of ways. The first is of the additive (or linear) type which, under literally uniform conditions, normally proceeds at a uniform rate of simple interest. In this sort of growth the product itself does not grow. The classical examples in organisms other than trees are the growth of such parts as shell, horn, and hoof (17). In the tree the product of this phase of cambial activity is, effectively, secondary wood. Its production is observed as the radial growth of the internode and its specific increment is referred to mean internodal cambial area, Ac.

The second kind of cambial growth is of the multiplicative (or logarithmic) type which, under uniform conditions, normally proceeds at a constant rate of compound interest. The important characteristic of this sort is that the product itself grows. In the simplest organisms virtually all cellular growth is of this replicative type but morphological complexity is inevitably accompanied by correspondingly large and numerous components of additive growth.

In the present instance growth of the multiplicative type appears in the production of new cambium through cambial activity. This aspect obviously has its relation to increase in girth.

The cellular basis of the distinction between additive and multiplicative cambial growth is simple in principle. The additive growth producing the secondary wood originates in cambial divisions occurring in the periclinal plane whereas the multiplicative growth, augmenting the complement of cambial initials as the axis enlarges, originates in divisions of cambial initials occurring in the anticlinal plane. Until the work of Bannan *et al.* (1–9, 24) revealed the remarkable proceedings involved in this part of the growth process and demonstrated how far from simple is the biological control of this function, it had been supposed that the number of annual anticlinal divisions might be, relatively to the periclinal, very small indeed, being in fact merely the minimum number required to produce the observed increment of fusiform initials. But Bannan has proved that in reality there is an astonishingly high rate of anticlinal division accompanied by an almost equal rate of elimination of the derivatives. This is achieved, apparently, by the selection of a small minority of survivors probably through nutritional and metabolic competition. The great majority fail to persist as cambial initials. Thus the intercellular conditions governing augmentation of the cambium rest upon the principle of nutritional competition between cambial cells. This principle entails maximum activity upon the average surviving cell of the cambium whereas the alternative principle of maximum mechanical efficiency (achieved through the use of the minimum possible number of divisions) would put a premium upon minimum activity of the average meristematic cell. We are inclined to agree with Huber (15) that this demonstrated mode of organizing cambial growth is the most probable source of the cambial stability upon which depends the phenomenal durability and longevity of trees.

Evaluation of Specific Annual Increment

Having considered the biological origin and significance of the two kinds of annual, specific growth increment we must next give them concrete expression. The approximation to the area of the internodal cambium that we adopt as the basis for specific growth is the arithmetic mean of the initial and final areas in any given year's growth. The mensurational expression for the internodal mean cambial area then evidently is:

$$Ac = \pi L(r_n + r_{n-1})$$

where r_n and r_{n-1} are the lengths of the corresponding radii of contiguous annual rings measured from the center of the pith and L is the length of the internode.

It is necessary also to state the terms in which the products of annual cambial growth are to be computed. The annual increment of wood manifestly must be given in terms of wood volume if increment is to be converted to specific increment by reference to unit cambial area as the measure of that which is active in growth. The only alternative, that of ring width, would

constitute a hopeless incongruity with an irrational and biologically valueless result. The adoption of cambial area as a measure of that which is active in growth is thus definitive for the evaluation of specific increment. The derivative formal relations with which we shall be concerned for the most part are the inevitable consequences of this adoption. Accordingly, the annual specific wood volume increment, the additive form of the annual specific growth by the cambium, is expressed for mensurational purposes:

$$S.I.V. = \frac{\Delta V}{Ac} = \frac{L\pi\,(r_n + r_{n-1})\,(r_n - r_{n-1})}{L\pi\,(r_n + r_{n-1})} = (r_n - r_{n-1})$$

and is manifestly equal numerically to the width of the annual ring. This simple and obvious relation is the formal justification for our previous use (13) of ring width as a numerical evaluation of growth activity even as it is the general reason for the choice of ring width as the measure of growth rather than ring volume wherever the activity rather than the amount of growth is in question.

The annual increment of cambial area clearly must be given in terms of cambial area. The specific increment will then be the area of new cambium formed in a given internode in a given year per unit area of growing internodal cambium. This, the multiplicative form of specific cambial growth, will be expressed:

$$S.I.Ac. = \frac{\triangle Ac}{Ac} = \frac{L2\pi\,(r_n - r_{n-1})}{L\pi\,(r_n + r_{n-1})} = \frac{(r_n - r_{n-1})}{\frac{1}{2}\,(r_n + r_{n-1})}.$$

Again the measure of specific growth is a function of ring width and is in fact the ratio of the width of the ring to the mean radius of the ring measured from the center of the pith. This will evidently be a small quantity, tending to become smaller at every internode as the diameter of the tree progressively increases. Its importance derives from its biological significance as the form of growth basic to the characteristic growth properties of the tree rather than from its numerically insignificant contribution to the mass wood growth of the tree.

3. *Annual Wood Volume Increment and Its Parameters*

In the preceding subsections we have considered specific growth of the internodal cambium and its numerical evaluation in *Pinus resinosa*, the formulation being at every step determined by the governing biological desiderata. We turned to the geometry of the tree only to find numerical expressions for the two forms of specific cambial growth that biological considerations led us to recognize as significant. In one of these, the annual specific increment of cambial area, we were concerned entirely with the two dimensions of the cambium. In the other, the annual specific wood volume increment, we were induced to adopt the volume of the internodal wood as the measure of the annual amount of radial growth partly in order to derive a real and rational measure of specific increment by referring the volume of annual internodal wood growth to the area of the internodal cambium that produced it. This yielded, necessarily in terms of internodal ring width, a specific annual increment of wood which thus becomes our measure of cambial growth activity in the production of wood.

The most useful single result of the foregoing analysis is the very simple conclusion that ring width is the numerical equivalent of a specific increment and hence is an appropriate measure of annual growth activity. This advantage of outlook having been gained, however, it is manifestly desirable to carry the analysis of wood volume increment a step farther by resolving the two dimensions of the cambial area into internode length, L, and cambial circumference, C. The former is a measure of apical growth and, as we have shown in subsection II. 1, under appropriate conditions it is equivalent to a measure of apical growth activity because its variations correspond to those of ring width which is, as we have demonstrated, a measure of specific growth. Cambial circumference, on the other hand, is obviously related to growth through increase in girth arising from the production of new cambium by the existing cambium, the activity measure of which is specific increment of cambial area. But in any given internode the length parameter is constant and cambial area varies only with circumference in passing from 1 year's growth to another. Under these conditions, which are essentially those of a type 2 sequence, a valid specific increment of cambium can be computed in terms of circumference. This further simple conclusion has a manifest application in growth studies, the primary data for which depend upon second type sequences such as those yielded, for instance, by the use of an increment borer.

This further breakdown of the component factors resolves internodal wood volume increment into the three geometrical parameters ring width, cambial (or ring) circumference, and internode length. Under appropriately chosen conditions each of these has its biological equivalent and affords a valid measure of a particular growth activity. The product of the activities thus evaluated represents the whole annual wood growth of the axis taken internode by internode.

It will be convenient to give the mensurational expressions for each of the several quantities even though they are obvious.

Wood volume increment.— Geometrically this is the volume of the internodal annual ring the expression for which is:

$$V = \pi L(r_n + r_{n-1})\,(r_n - r_{n-1})$$

and which represents the amount of secondary wood production.

Internode length.—The apical growth component represented by the symbol L. It is the length of the internode measured in units corresponding to those in which the linear dimensions of the cambial system are given.

Mean circumference.—This is the arithmetic mean circumference of the internodal cambium at the beginning and at the conclusion of a year's growth. It is numerically equal to the replicative growth component of the cambium wherever, as within a given internode, the length parameter is constant. Put in another way, the mean cambial circumference is numerically equal to the mean cambial area per unit length of internode and is thus the simplest formulation of the amount of replicative cambial growth abstracted from all other growth functions. The expression is:

$$C = \pi(r_n + r_{n-1})\,.$$

Under appropriate conditions, C can obviously be used to replace cambial area, Ac, in computing specific increment since the specific increment of cambial area and the specific increment of cambial circumference are numerically equal and must have the same distribution in the tree. It should also be noted that the *annual increment of cambial circumference* is 2π times the specific wood volume increment (ring width). Its distribution in the tree therefore must follow exactly that of the latter measure which has been considered in our first paper (13) and therefore need not be brought to account in this communication.

Ring width.—This is the additive cambial growth component, the annual radial increment which is numerically equal to specific wood volume increment. The expression is:

$$\Delta r = (r_n - r_{n-1}) .$$

III. Results for a Typical Tree

1. *Internodal Wood Volume Increment and Its Parameters*

Wood Volume Histogram

Fig. 1 is a diagram of a kind first employed for the survey of internodal ring systems in our previous paper (Duff and Nolan (13, Figs. 5, 7, 9, 11)) where its composition and purposes are fully described. The present diagram records the internal design of the rings in terms of ring volumes for tree K52.1, which previously served as the type tree in the comparative study of ring width. This tree was one in which living, active branches extended down the axis for about half its length. In this way the tree represented the closed or forested type of growth rather than the open grown type in which the tree of this age is branched to the ground.

Fig. 5 of the previous paper gave the data for ring width that correspond to these present data. The general character of the sequences of internodal ring volume is discernible by close inspection of Fig. 1 but we shall record below, for clarification of detail, certain sequences in the form of graphs which correspond in principle to those represented in Figs. 3 and 4 of the earlier paper.

Fig. 1 shows that what we have called the "pattern" of a family of ring width sequences for a given tree is manifest also in the data for ring volume. However, the great regularity of the data for ring width is not reproduced here; instead we find the pattern somewhat perturbed. The perturbing influence is manifestly the introduction of the factor of internode length into the computation of ring volume.

The activity of the yearly wood growth measured as internodal ring width is a purely cambial activity and it is distributed in the tree's axis with remarkable regularity. We have sought the origin of the regularity in the response of the cambium to factors of internal origin which we believe to be metabolic or nutritive and concerning which we shall have more to say in future communications. These factors are held to be localized as to origin in the active

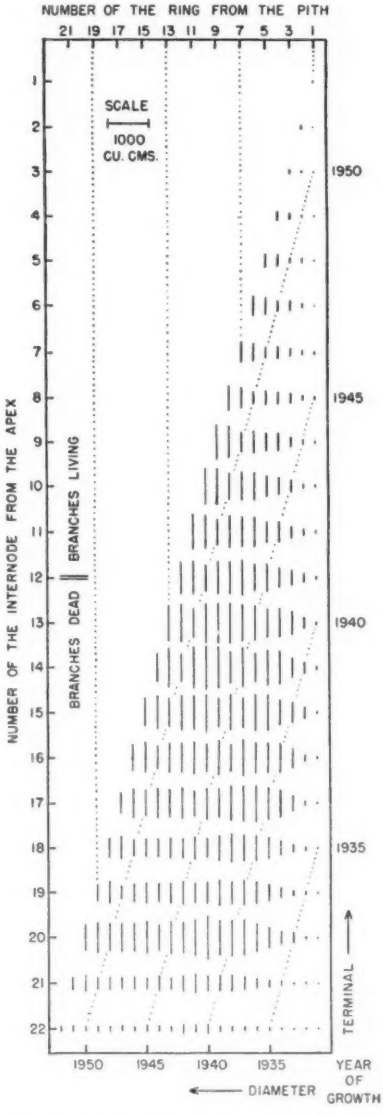


FIG. 1. Histogram recording the distribution of wood volume increment in the tree. Apex of the tree at the top, pith on the right, and the outer and most recently formed internodal rings on the left. The wood volume of each internodal ring is represented by the length of a short, vertically drawn line placed at the ring's position in the tree. The scale of volume is given in the figure. Type 1 sequences run obliquely from upper right to lower left and the course of certain ones is indicated by dotted construction lines. Type 2 sequences run horizontally. Type 3 sequences are vertically oriented and certain ones have their course indicated by construction lines. Standard plantation tree K52.1.

foliage of the crown. In any tree of this species, but particularly in a tree growing under closed conditions with a correspondingly limited crown, it is clear that the effective *locus maximus* of the internal factors is never stationary in respect of any given axial internode but year after year shifts its relative position first toward, then past, and finally away from that internode as the tree grows. The nutritional status of the internodal cambium changes correspondingly, at first improving, then after maximation, ever more slowly deteriorating. The *activity* of the cambium, measured as ring width, changes accordingly (13, Fig. 4).

In marked contrast, it is obvious that the annual *amount* of wood growth (measured as internodal ring volume) does not depend upon the activity of the cambium alone but upon the length of the internode also. The latter parameter of internodal growth manifestly assumes a fixed magnitude in the year of the internode's origin. Apical growth in that year is conditioned by factors operating in a particular combination which is quite different from and independent of the successive combinations that in subsequent years influence cambial activity in the same internode. The effective determinants of internode length thus operate uniquely each year upon the apical growing point. This meristem is not subject to a gradual change in its relation to internal nutritional factors of foliar origin as the internodal cambium is. On the contrary, the apical meristem maintains a substantially constant spatial — and hence supply — relation to the foliar growth factors because of its uniformly apical position. The product of apical growth is, accordingly, unaffected by the influence that induces "pattern" in the products of cambial growth and this factor's systematic effect is absent. Accordingly, extrinsic factors of random incidence act upon the apical growing point as upon a metabolically stable rather than a drifting meristem with the result that, over the years, the sequence of internode length reflects the action of the extrinsic factors with a higher degree of fidelity, though in a more complex manner, than the wood rings unless the latter are formulated as a type 3 sequence for the express purpose of evading "pattern". Extrinsic factors of random incidence thus become the dominating determinants of apical growth when this is measured as internode length.

The controlling circumstances of the two meristems are evidently profoundly different as we have already stressed (13, pp. 500–507). When, therefore, unpatterned internode length, the product of apical activity, becomes the coefficient of patterned ring width, the product of cambial activity, in the computation of internodal ring volume, the resulting volumes in their several sequences are necessarily impressed with the random variations of internode length and exhibit corresponding irregularities. This effect is all the more prominent from the obvious fact that internode length is so much greater numerically than ring width. The degree of difference often exceeds two orders of magnitude. The force of this point, and indeed the relative importance of all three geometrical parameters of internodal wood growth, is best appreciated from a graphical presentation of the data such as that given in Fig. 2.

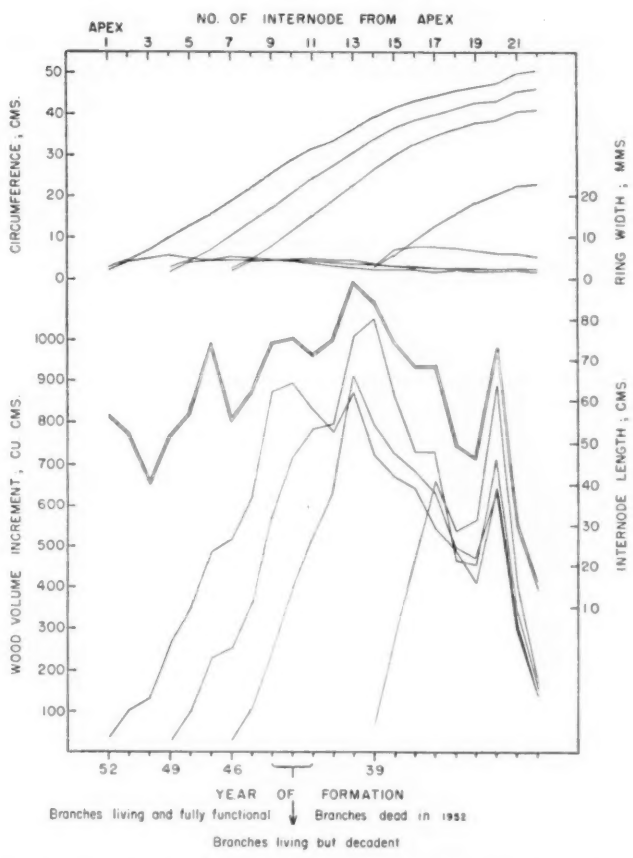

APEX
NO. OF INTERNODE FROM APEX
1 3 5 7 9 11 13 15 17 19 21
CIRCUMFERENCE ; CMS.
RING WIDTH ; MMS.
WOOD VOLUME INCREMENT ; CU CMS.
INTERNODE LENGTH ; CMS.
52 49 46 39
YEAR OF FORMATION
Branches living and fully functional
Branches dead in 1952
Branches living but decadent

FIG. 2. Four arbitrarily chosen first type sequences of wood volume increment (below) plotted according to the first mode, in which the abscissal scale has a mainly positional significance with the apex of the tree at the graphical origin. Each type 1 sequence traces the volume of the internodal wood ring of a given year from internode to internode down the axis of the tree. A subordinate time scale marks the year in which each internode originated. With the wood volume increment sequences are plotted (above) the geometrical parameters of internodal wood volume – cambial circumference and ring width also as first type sequences. The scale of the latter is 10 times that of the former. In the center of the diagram the third geometrical parameter, internode length, is plotted to the scale of cambial circumference and wood volume increment. Standard tree K52.1.

Wood Volume Increment Sequences of the First Type

In Fig. 2 we have plotted the first type sequences of wood volume increment for tree K52.1. These are the sequences which in the previous figure run diagonally through the histogram and are those which trace the annual wood volume increment, for each year of growth separately, from internode to internode down the tree. In order not to confuse the diagram with too many

lines, four only of the 21 possible sequences of the first type have been represented in the figure. These are the sequences for the years 1952, 1949, 1946, and 1939 and were arbitrarily chosen. All the others conform closely to these.

We have already noted from the histogram that the first order sequences manifest pattern. The character of the distributive pattern in the tree is exhibited in detail in Fig. 2. There is evidently a basipetally progressive increase in the numerical value of the distal terms of the sequence as there was also in the pattern of internodal ring width (13, Fig. 3), but for ring volume the increase continues for about twice as many internodes (9 or 10) down the tree as that for ring width (4 or 5). It is therefore obvious that ring volume continues to increase progressively in a sequence of type 1 in which ring width is diminishing. This combination is associated in this particular tree with an irregular, basipetally progressive increase in length of the upper 13 internodes but it would be possible geometrically to combine increase in ring volume with decrease in ring width even though internode length did not increase, as in some trees (see tree K47.5 *infra*) it does not. The maximum wood growth *activity* (measured by ring width) thus occurs in an internode considerably higher in the tree's axis than that in which the maximum *amount* (measured by ring volume) of internodal wood growth occurs. This distinction accentuates the antithesis between measures of activity and measures of quantity in studies of growth. It also illustrates the fact that in a given tree the sequences of two growth measures which are significant in different contexts do not exhibit identical patterns. Most measures of biological growth have a similarly limited validity.

In order that the determinants of internodal wood ring volume may be made visually manifest three other sets of graphs are combined with the first type wood volume increment sequences in Fig. 2. The upper graphs are the type 1 sequences of internodal ring width and internodal ring circumference. Both these are derivatives of cambial activity. The central graph is the sequence of internode length, a derivative of apical activity. All three are plotted on corresponding abscissal scales both as to space and time. The ordinate scales of circumference and internode length are plotted in centimeters, that of ring width in millimeters. Their algebraical product yields the annual wood volume increment in each internode and accordingly the pattern of their sequences jointly determines the pattern of the corresponding sequences of wood volume increment. Clearly, however, the geometrical relations by themselves would be trivial; it is their biological significance that gives them interest. Each of the geometrical parameters has been shown in subsection II. 3 to have its proper and distinct biological significance to which we shall return after examining the geometrical and numerical relations.

The major geometrical determinants of wood volume increment can be identified readily by inspection of Fig. 2. Regarding first the sequences of ring circumference, ring width, and internode length, the most prominent characteristic of the data is the smallness of the numerical values and the

narrowness of the range of variation in ring width when contrasted with the other two as they are plotted. This discrepancy has already been referred to above. But ring width was actually plotted in Fig. 2 on a 10 times greater scale than the other parameters, so that on equal scales the graphs for ring width would for practical purposes be straight lines parallel to the abscissal axis. It is manifest, accordingly, that variation in ring width contributes relatively little to the determination of sequential variation in wood volume increment but enters into it as an almost constant factor of small dimensions. It follows that the dominant numerical determinants must be ring circumference and internode length. These are the components of internodal cambial area and their joint dominance in determining wood volume increment simply means that differences in the amount of internodal cambium are numerically more important than the corresponding differences in cambial activity.

Fig. 2 shows that in every sequence the volume of the internodal ring increases in rapid progression downward from the apical internode of the year of origin. The rising arms of the plotted sequences clearly owe their substantially regular form mainly to the corresponding, regularly rising sequences of internodal ring circumference, which thus appears to be the major determinant of wood volume increment in the upper zone of the tree. Large reverse variations of internode length induce recognizable corresponding variations of wood volume increment in the upper or distal portions of the sequences, but the slightness of the induced, relative to the size of the inducing, variations testifies to the comparatively minor influence of variance in internode length upon that of wood volume increment in the upper part of the tree. However, reading basipetally from the uppermost internodes in any recent sequence, the data show that after a certain point the sequential variation of wood volume increment in this tree approximates with every successive term more and more closely to the variation of internode length. The approximation is easily recognizable by inspection of the graphs at about the seventh internode and continues progressively to the 9th or 10th, from which point downwards wood volume increment manifestly varies in the closest accord with internode length. This point roughly corresponds in each sequence to the maximum of wood volume increment. Each recent sequence therefore traces along the axis a transition between states in which the major determinant of sequential variation in wood volume increment passes from ring circumference to internode length, that is, from cambial to apical dominance. The recent sequence also localizes the two states themselves, the former being characteristic of the upper and the latter of the lower zone of the tree.

In this tree the recent, first-type sequences of ring circumference are curves of very flat sigmoidal form (Fig. 2). They pass their point of inflection and enter upon the phase of declining slope at about the internode in which wood volume increment begins to vary in close accordance with internode length. It is also clear that the transition along the axis (involving several internodes) from whorls of living and fully functional branches to whorls of completely dead branches occurs at about the same position on the main axis (Fig. 2).

This spatial correspondence again relates the growth of wood to the complex of nutritional factors that originates distally in the foliated and metabolically active crown of the tree. The transitional maximum in the sequence of wood volume increment (i.e. amount of growth) occurs at a lower point on the axis than the corresponding maximum of ring width (i.e. activity of growth (13)). This may be attributable simply to the circumstance that the first type sequence of the former (Fig. 2) contains the progressively augmenting factor of circumference while the latter does not. It may be worth while to note, however, that the tree's geometry indicates that its metabolic correlatives are complex, as suggested above, and in particular that the nutritional factor conditioning growth must be regarded as involving the whole range of possible component types of metabolite from energy components to components of vitaminic and hormonal character, and is, accordingly, capable of differential effects upon growth.

We now construe the foregoing numerical and empirical relations into their biological and functional equivalents. The first conclusion regarding growth is that in the upper zone of the tree the dominant growth activity is the production of new cambium. In this zone wood volume increment increases basipetally in the growth of every year and the major geometrical determinant of the first type sequence of wood volume increment in this zone is rapidly increasing circumference (Fig. 2). The cambial process characteristic of increase in girth we have already found to be the anticlinal division in which the product survives as a normal fusiform cambial initial and neither degenerates nor maturates (Bannan (1 to 8), Whalley (24), Bayly (9)). Only divisions of this sort lead to net increase in cambial circumference and hence to an increase in the the cambial area of the internode. The annual increment of cambial area is the measure of the amount of such internodal growth and the measure of its activity is the specific increment of cambial area. In subsection II. 2, we distinguished this type of growth activity as the multiplicative growth par excellence of the cambium. Accordingly, the greatest activity of multiplicative growth would appear to occur in the youngest (and smallest) internodal cambia. A further consideration of internodal cambial dimensions and of the specific increment of cambial area will be given in a following subsection.

The second conclusion regarding the distribution of the biological parameters of wood growth is that in the lower part of tree K52.1 where the internodal cambia are older, bigger, and more remote from nutrition of foliar origin and where also circumference increment has become relatively small and still grows progressively smaller with descent of the tree, the sequential variations of wood volume increment come automatically to be dominated by the terminal growth parameter:internode length. Thus the dominating influence of the apical meristem upon sequential variation is necessarily effected at times antecedent to the cambial activities which produced the later wood rings in these lower internodes. We have seen that internode length is an empirical index of apical activity and the irregular fluctuations of this

index have been attributed to the effects upon apical activity of the random incidence of unstable, extrinsic factors of climatic origin (13, p. 500 *et seq.*). Similarly, the major topographical feature (i.e. "configuration") of the plot of internode length are believed to stem from the stable external factors arising out of the properties of the site and stand (13, pp. 496–497). If so, then in contrast to the conditions of cambial growth, all the prominent sequential variations of yearly terminal growth would appear to be externally induced. There is little evidence of modification by internal factors. Evidently the apical growing system is an important effector mechanism in the control of wood growth by extrinsic factors.

The data indicate that in a forested red pine of about 25 years onward, such as K52.1, the annual internodal wood volume increment comes to be dominated numerically by the consequences of past apical activity over an increasingly large part of the tree. By contrast, the amount of the wood production that is dominated by cambial action is restricted to a more or less uniform upper zone in such a tree. This zone is most closely associated structurally with the crown of foliage and we consider that it is associated physiologically with metabolic products of the foliage and buds. This upper zone represents a continually declining proportion of the whole forested tree. The condition of apical preponderance in the variations of the annual wood production is reached first in the lowermost parts after a comparatively few years' growth and appears to extend upward in the main axis at a rate substantially corresponding to the rate of the tree's vertical growth and the progressive failure of the lower living branches. As we shall see in a later subsection of this paper (IV.1), the wood volume increment of an open-grown tree possessing active foliage at ground level is, on the other hand, cambium-dominated over the entire axis.

2. *Dimensions of the Internodal Cambium and Their Determinants*

The reason for giving special consideration to the magnitude of the internodal cambium at this point of the discussion lies simply in its being the thing that is active in secondary wood production and its magnitude therefore is the only proper basis for the derivation of the specific activity of secondary wood growth. We have shown in the preceding subsection how the important parameters of annual wood volume increment are the two components of our measure of the magnitude of the internodal cambium, that is, of mean cambial area. The two components are, of course, internode length and mean cambial circumference. The latter derives its importance from its relation to growth in the sense of increase in girth and thus to the multiplicative growth of the cambium. The former commands attention as the index of apical growth activity.

Histogram of Mean Cambial Area

In subsection II.2 we defined our measure of cambial area as the arithmetic mean of the initial and final areas in any given year's growth. Values so computed for tree K52.1 are given in the form of a histogram in Fig. 3.

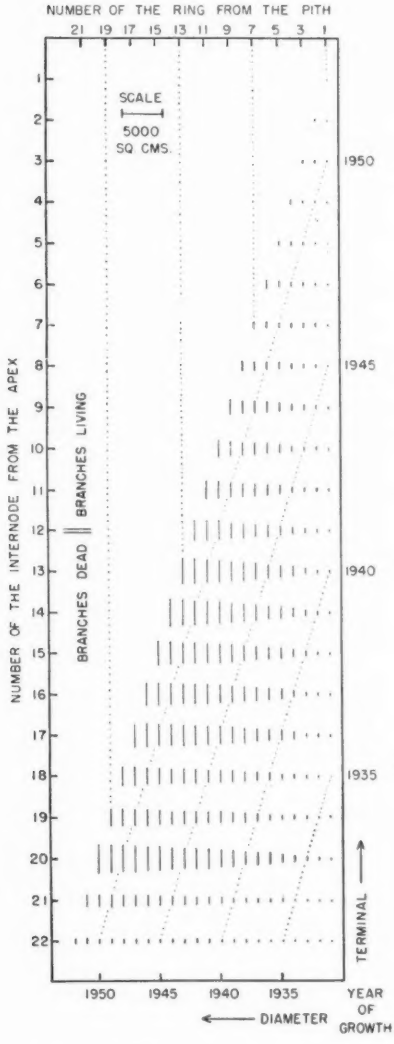


FIG. 3. Histogram of mean cambial area, recording the distribution of this measure of cambial size in the tree. The diagram corresponds to FIG. 1 in every formal way but records a different measure of growth. Standard tree K52.1.

The data thus displayed exhibit points of strong similarity to the corresponding data for wood volume increment (Fig. 1). The similarity derives from the fact that internode length is a major factor in both data and hence in passing sequentially from one internode to another the factor of internode length induces in both data strongly similar variations.

The differences from wood volume increment appear most evidently in the horizontal or type 2 sequences which proceed from pith to cambium each in a single internode. In the data for wood volume increment somewhat fluctuating values appear following a rapid increase from the pith outward for four or five rings. The corresponding sequences of mean cambial area, on the other hand, exhibit a gradual, uninterrupted increase from pith to cambium. This distinction reflects the absence from the mean cambial area datum of the factor of ring width.

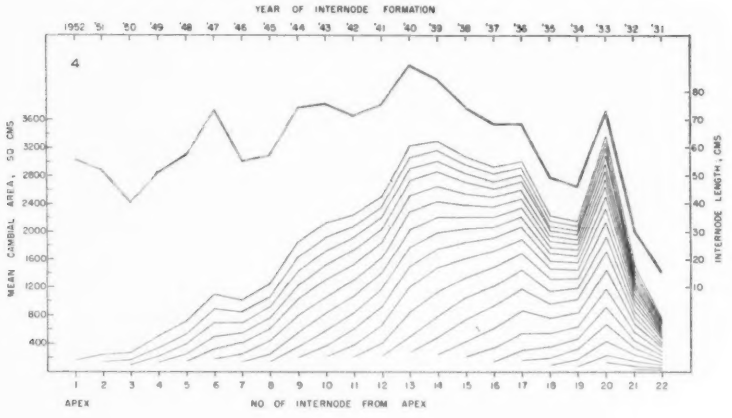
Though the general features of the distribution within the tree of the amount of cambium are clear from inspection of the histogram, closer consideration requires the plotting of the sequences in graphic form. These are given in Figs. 4, 5, and 6.

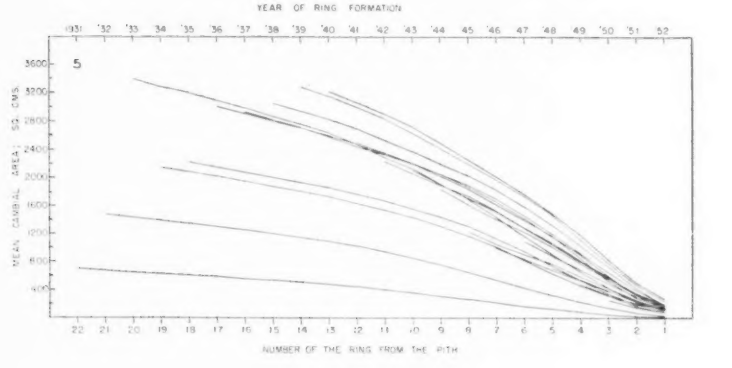
First Type Sequences of Mean Cambial Area

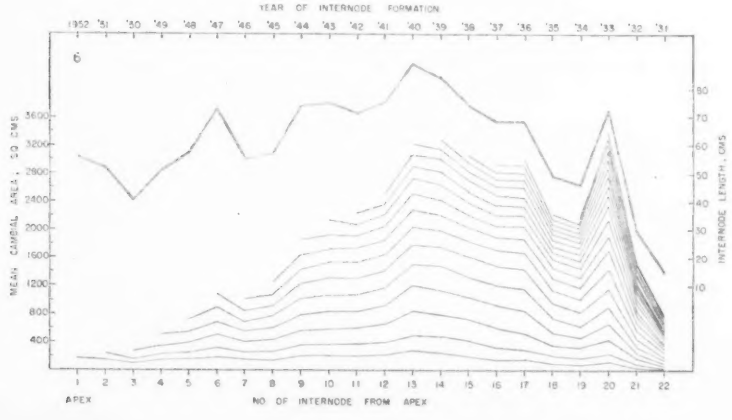
The first type sequences for tree K52.1 appear in Fig. 4 and with them are plotted the sequence of internode length for this tree. Comparison with Fig. 2 shows the extent to which the present data conform to those for wood volume increment. The similarities could hardly fail to be considerable in view of the fact that the numerical difference between the two bodies of data lies in the derivation of wood volume by multiplication of cambial area by the relatively steady values of ring width. As with wood volume increment, so here, the sequential variations of the first type sequences are determined in the upper zone of the tree mainly by the sequential variations of cambial circumference and in the lower zone by internode length. In general the observations that applied in the preceding subsection to the sequences for wood volume increment apply here to those for mean cambial area.

Second Type Sequences, Mean Cambial Area

Sequences of type 2 are given in Fig. 5. Their form, apparent from the histogram, is that of a family of gently downhill curves, convergent, but maintaining throughout their length their proper differences of pitch and slope. The smooth form of each sequence derives from the fact that all its terms lie within a single internode. In this way the factor of internode length enters into the computation of the terms of any given sequence as a constant. The forms are thus a simple derivative of the forms of the corresponding values for cambial circumference and these, as we have seen, are very regular. On the other hand the value of internode length is different for each type 2 sequence of cambial area and these differences are responsible for the distribution and spacing of the sequences in Fig. 5. Those of highest pitch are the sequences lying in the longest and those of lowest pitch in the shortest internodes.


YEAR OF INTERNODE FORMATION
1952 '51 '50 '49 '48 '47 '46 '45 '44 '43 '42 '41 '40 '39 '38 '37 '36 '35 '34 '33 '32 '31
4
MEAN CAMBIAL AREA, SQ CMS
INTERNODE LENGTH, CMS
APEX
NO OF INTERNODE FROM APEX


YEAR OF RING FORMATION
5
MEAN CAMBIAL AREA, SQ CMS
NUMBER OF THE RING FROM THE PITH


YEAR OF INTERNODE FORMATION
6
MEAN CAMBIAL AREA, SQ CMS
INTERNODE LENGTH, CMS
APEX
NO OF INTERNODE FROM APEX

The functional significance of this diagram is simply that the internodal cambia, whether they are big because of lying in a long internode or small because they belong to a short internode, all alike increase in area, year after year, but they do so more and more slowly. The highest rates of increase are thus found in the youngest internodes, which are at every stage of growth the internodes of the contemporary upper zone of the tree. As we have already seen, this explains the dominant effect of cambial circumference as a determinant of wood volume increment in the upper zone of the tree.

Sequences of the Third Type, Cambial Area

These are given in Fig. 6 together with a plot of the sequence of internode length. Again the sequential variations of cambial area conform to those of internode length because both sequences pass from one internode to the next in their successive terms. But they conform in different degrees depending upon their distribution in the tree. Again it is evident that the conformity becomes closer as the lower zone of the tree is approached and entered. This is true of each sequence separately but, of course, more particularly of those which have the largest proportion of their terms corresponding to rings of recent date. The later terms of such sequences change but slightly by reason of increase in circumference from one internode to the next (Fig. 5) and their variation thus depends most heavily upon variation in internode length. There is no evidence of progressive increase or decrease in the terms of any type 3 sequence of cambial area.

Mean Cambial Circumference

First type sequences of this geometrical growth parameter appear in Fig. 2, where four representative sequences are plotted, as are all the graphs in that figure, according to the first mode. This plot corresponds in mode of plotting to that of Fig. 3 in our first paper (13) and is one in which the abscissal axis retains both positional and temporal significance but the former is the more important in this mode of plotting. Fig. 7 gives all of the 21 such sequences which tree K52.1 afforded. The graphs are this time plotted according to the second mode, which corresponds to the plot of Fig. 4 of the first paper. In this mode of plotting the abscissal axis is relieved of its exact positional significance for all sequences except the longest and most recent, and the sequences are aligned so as to make the graphical origin represent the year of origin of every sequence (and internode). This mode of plotting is designed

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FIG. 4. Diagram of the entire family of first type sequences of mean cambial area plotted according to mode 1 together with internode length in order to demonstrate differences in the degree of conformity between corresponding sequences of cambial area and internode length in different parts of the tree. The scales are equivalent. Standard tree K52.1.

FIG. 5. Diagram of the entire family of second type sequences of mean cambial area. Standard tree K52.1.

FIG. 6. Diagram of the entire family of third type sequences of mean cambial area plotted according to the first mode together with internode length. The scales are equivalent. Standard tree K52.1.

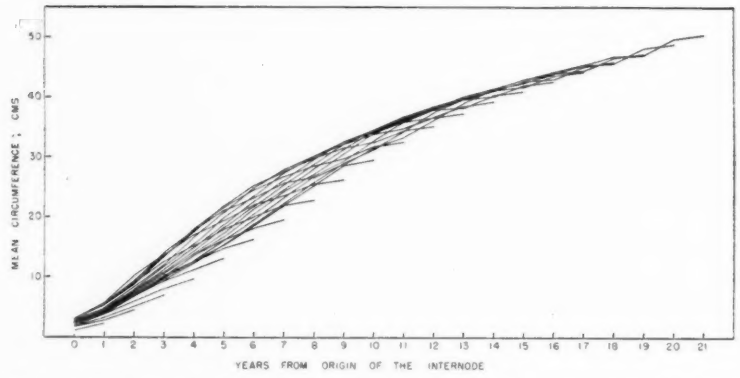

FIG. 7. Diagram of the entire family of type 1 sequences of mean cambial circumference plotted according to the second mode in order to stress their homogeneity of form. Standard tree K52.1.

to stress the forms of the sequences so that their similarities and differences may be contrasted as clearly as possible. The family of first order sequences of mean cambial circumference thus appears in Fig. 7.

Clearly the family exhibits a strong unity of form as a series of flat sigmoids. The sequences are not coincident but the range of homologous intersequential differences is small. The range is very small in the early years of internodal growth increases to a maximum in about the fifth year and narrows down again in the later years. In the earliest years of internodal growth the range of the intersequential differences involves internodes of corresponding age in all stages of the tree's development from the little sapling hardly bigger than a transplant to the young, fruiting tree. In the latest years only the older developmental stages are involved. We shall show in subsection IV.2 that homologous intersequential variation is narrower still in an open grown tree on a good site but is much wider in a forested tree on a poor site.

The conformation of the family of first type sequences when thus plotted in the second mode suggests that, for a tree such as this, a maximum, mean cambial circumference for an internode of every age would be deducible quite simply as the upper limit of the family at every internodal point throughout the series of internodal ages. If these points were joined by a line the resulting graph would be a composite of many sequences, each sequence rising in succession to overtop its predecessor and thus to establish the upper limit of the family for, usually, a single point representing one internode. This line denotes the maximum or limiting amount of multiplicative growth of an entire tree of this species and age-group under the conditions of a single site and genetic hereditament.

Such a datum as the above should be useful for a variety of comparative purposes. For instance, it could be used to compare growth (or the expectation of growth) in a population of trees selected for uniformity but planted in

contrasting densities and/or upon contrasting sites. This would be the equivalent of using multiplicative cambial growth as a criterion in the evaluation of site and stand. Since multiplicative cambial growth is the function that everywhere underlies the integral growth of the tree, it would seem appropriate for it to be used as the growth criterion in something as important and difficult as site evaluation. Again, this criterion could be used to compare growth amongst the members of an unselected maximally heterogeneous population established in a uniform stand on a uniform site. In this instance multiplicative cambial growth would be used as the criterion for the evaluation of different genetic hereditaments functioning under specific, approximately uniform conditions. Once more the criterion would appear to be appropriate. We have calculated maximum, mean cambial circumference for trees for which we have suitably long sequences and later in this paper we propose to compare in terms of this criterion the three trees which have contributed the illustrative data that we are here chiefly considering.

3. Increment of Cambial Area

The distribution of internodal, cambial area in the tree has been fully described in the preceding subsection. As the numerical measure of the thing that is active in wood growth, and therefore the basis of reference for specific growth, it is desirable to present as comprehensive a view of its distribution in the tree as possible. The growth of the cambium itself is one of the basic forms of replicative growth in the tree and its magnitude and distribution are correspondingly important. As a preliminary to considering the cambium's specific growth increment and its distribution we have thought that a histogram of the increment of cambial area should be recorded. The diagram is given in Fig. 8. This figure can be compared with the corresponding diagram for cambial area (Fig. 3), for wood volume increment (Fig. 1), and for specific wood volume increment (ring width) (13, Fig. 5).

The numerical differences between annual increment of cambial area and the other measures of the growing system or its products are evident enough. For example, the difference between annual increment of cambial area and wood volume increment lies in the absence from the numerical value of the former of the ring width parameter. Again, the numerical difference between the annual increment of cambial area and that of cambial circumference lies simply in the absence of the internode length parameter from the numerical value of the latter. The effect of these differences upon distribution can readily be deduced and is observable in the several diagrams. But the comparison of greatest interest is that between the distribution of annual increment of cambial area (Fig. 8) and of cambial area itself (Fig. 3). Comparing the type 2 sequences, for instance, it is obvious that whereas mean cambial area forms a gradually but continually augmenting series, increment of cambial area forms a diminishing series except for three or four initial terms that rapidly increase, thus giving evidence of pattern and therefore of the influence of the intrinsic, systematic factor (13). The form that is to be expected in

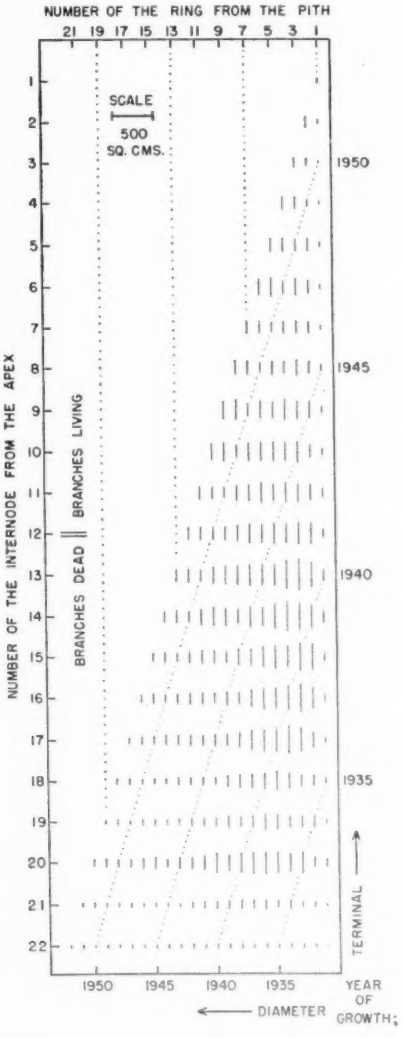

FIG. 8. Histogram of annual increment of cambial area, recording the distribution in the tree of this measure of cambial growth. The diagram corresponds to FIGS. 1 and 3 in every formal way but records a different measure of growth. Standard tree K52.1.

sequences of specific increment of cambial area can thus be deduced roughly from such comparisons but we shall nevertheless present the data explicitly in a following subsection.

4. Increment of Cambial Circumference

The chief interest in treating a function of cambial circumference as a measure rather than as a parameter of cambial growth lies in the fact that this measure contemplates only the kind of growth that underlies increase in girth. As we have already seen, this is the replicative kind of cambial growth in which anticlinal divisions of cambial initials lead to the augmentation of the existing complement of these meristematic cells and so to the growth of the cambium.

In subsection II.2 it has been shown that the distribution in the tree of annual increment of cambial circumference must be precisely the same as that of specific wood volume increment (ring width) because the former measure is simply 2π times the latter. This distribution has already been fully described and its meaning considered exhaustively (13). The subject need not be recapitulated. It is interesting to observe, however, that the very simple, numerical relation between the annual specific wood volume increment (ring width) and cambial circumference increment as well as its corollary, the identical sequential distribution of these two measures in the tree, bespeak the dependence of the physiological function underlying the former measure upon the function underlying the latter. We have referred to this elsewhere. We may also remark that the strict correlation between the increment of cambial circumference and specific wood volume increment shows that the former must, like the latter, be strongly controlled by the intrinsic metabolic-nutritional factor of foliar origin which we identified in action in our previous paper (13). In a later section of the present paper it will appear that the specific increment of cambial circumference (and area) exhibits no evidence whatever of control by this intrinsic factor but that a factor of quite different origin and character must be postulated as an intrinsic control.

5. Specific Wood Volume Increment

The significance and derivation of this growth function have been considered in subsection II.2 where we show that the specific wood volume increment is the best available measure of the activity of annual, internodal wood growth and that it is numerically equal to ring width. Since the data for ring width have already been presented and considered exhaustively, specific wood volume increment need not be discussed in full here and no presentation of data is required. The data for the type tree K52.1 are given in Figs. 2 to 5 inclusive in our first paper (13). All that is necessary to render these figures immediately conformable to present considerations is to convert units of the growth measure from ring width in millimeters to specific wood volume increment in milliliters per square centimeter of mean cambial area. This merely requires division of the ordinate scale graduations by 10.

The reader is referred to the preceding paper also for the full discussion of wood growth there given not only for the type tree but also for comparison and supplementary trees which grew under contrasting conditions. The only addition that need be made here to the published discussion is simply to add the observation that everything that was there written about growth in terms of ring width derives its validity from the fact that ring width is the best measure of the activity of the additive type of cambial growth.

6. Specific Increment of Cambial Area

This growth function has already been considered in Section II as to both its significance and its derivation. It is a general activity measure of the replicative form of annual, cambial growth in which the product itself grows. The cellular basis of this kind of cambial growth is an immense overproduction of new cambial initials by cell divisions in the anticlinal plane accompanied by the competitive selection of a small proportion of survivors which ensure the maintenance and increase of the internodal cambium. This is a function of fundamental importance to the organism as a whole because it is by this means alone that the cambium itself is maintained and augmented. Linked to this kind of cambial activity is a second kind, not replicative because the cellular product does not continue in growth, but a form of additive growth. The latter is the cambial production of secondary tissues, which has just been considered in subsection III.5. For purposes of simplification this growth function is identified with the production of secondary wood. These two functions encompass the whole activity of the cambium. The latter activity having been considered above, the former must now be accounted for in this section of the paper.

The Automatic Effect of Increasing Cambial Size

The numerical basis of cambial growth has been defined as the mean cambial area. This measure of the growing system has also been considered in a preceding section and its sequential variation in different parts of the tree has been described. The derivative function is specific increment of cambial area, the distribution of which in the tree is shown in Fig. 9. When this figure is compared with Fig. 3, the histogram of mean cambial area, their inverse character is manifest at once. The big specific increments are produced by young (and small) cambia and the smaller increments by older (and bigger) cambia. Thus, as the cambium inexorably grows older (and bigger) its specific annual increment diminishes and the consequence is manifest in the first and second type sequences (Fig. 9). These are the sequences which are characterized by the property that each term is the growth product of an older (and larger) cambium than that of the antecedent term. In type 3 sequences, on the other hand, all the terms of a given sequence are the growth product of internodal cambia of equal age and roughly equal size.

At the close of subsection II.2 wherein the numerical evaluation of specific increment of cambial area was considered, it was shown that the expression

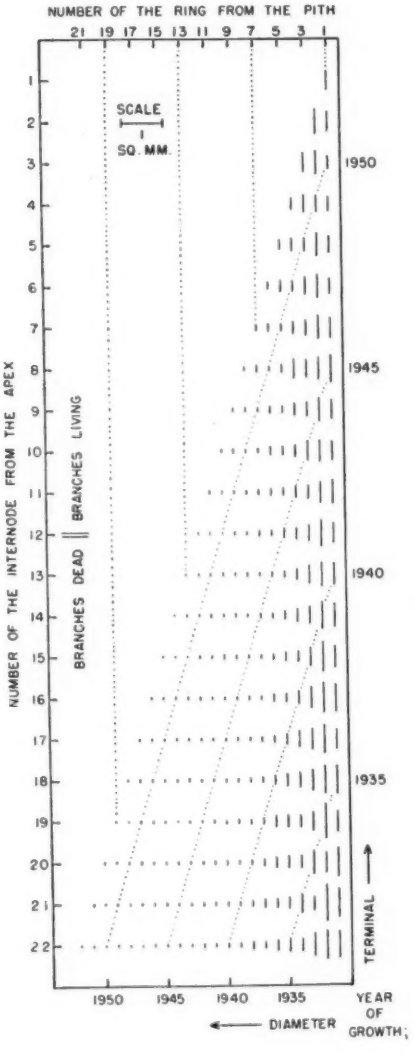

FIG. 9. Histogram of specific increment of cambial area, recording the distribution in the tree of this measure of cambial growth. The diagram corresponds to FIGS. 1, 3 to 8 in every formal way but records a different measure of growth. Standard tree K52.1.

for this function contained in the denominator the measure of the ring's mean radius from the cross-sectional center of the internode. It was also stated that for this reason the numerical value of specific increment of cambial area would diminish progressively as the tree steadily increased in diameter by growth. Regarding this question more closely, we have reached the conclusion that the highly regular distributive conformation of cambial growth increment is to be attributed as much to the purely mechanical or automatic consequences of variation in size as to the biological circumstances of annual growth in the tree. The progressive increase in the size of the internodal cambium is numerically more important to the result than the corresponding increase in age and overshadows any effect there may be of the latter factor upon specific cambial growth. The reason for the automatic effect is that the growth of the internodal cambium does not vary freely but only within quite narrow limits set by boundaries of time, space, environmental conditions, and the activity of physiological functions contributory to growth. In the tree some such boundaries are, for instance, the narrowly varying length of the growing season, the fixed length of an internode, and, at any site to which the species is at all well adapted, the narrow range of variation from year to year in growth-controlling conditions such as temperature and moisture. Even the amount of secondary wood production could function as a boundary determinant of cambial increment. By contrast with this restricted amplitude of variation in annual increment, the magnitude of the internodal cambium, to which specific growth is referred, is the indefinitely cumulative summation of growth. In any internode of a young tree of a species such as *P. resinosa* this summation is proceeding to a limit which is exceedingly remote. Accordingly, under favorable conditions we must expect to find that the restricted limit of increment is approached with considerable regularity, year after year, while cambial area, to which the increment is referred in the computation of specific growth, progressively increases. The cellular arrangements are such as to confirm us in this view: The great overproduction of cambial initials not only ensures the maximum activity of the surviving products to which we have drawn attention earlier but also thereby ensures that the limit of increment set by the boundary factors referred to above can be regularly approached.

To these attributes, which in greater or lesser degree characterize specific growth in all organisms (cf. 17), the distribution of specific increment of cambial area in first and second type sequences necessarily and closely conforms. This compulsion, arising simply but inescapably out of the difference in the mode of variation between the continuously progressive size of the thing that grows and the discontinuous and narrowly varying magnitudes of its annual increment, makes it inevitable that the distribution should take the form of a decline of gradually diminishing slope. We shall refer to this effect as the "automatic effect of progressively increasing cambial size". The fact that we refer to it the form of the first and second type sequences of specific increment does not mean that it is to be regarded as the unique determinant of the observed form but merely that it is the dominating

determinant. Other determinants are certainly involved but their effects are masked by that of the "automatic" factor so that it is difficult or impossible to distinguish them. But, as we shall see presently, the automatic effect is eliminated from sequences of type 3 so that the effects of other determinants of specific increment, both systematic and random, are disclosed and can be evaluated in these sequences.

First and Second Type Sequences, Specific Increment of Cambial Area

First type sequences are plotted in Fig. 10. In order that the figure might not be obscured by too many lines, only half of the tree's sequences are represented. The choice of the sequences for the years of odd number is arbitrary. The distributive conformation is manifestly very regular and very uniform from one sequence to another and the family of curves is demonstrably an approximation to the hyperbolic form. This characteristic regularity

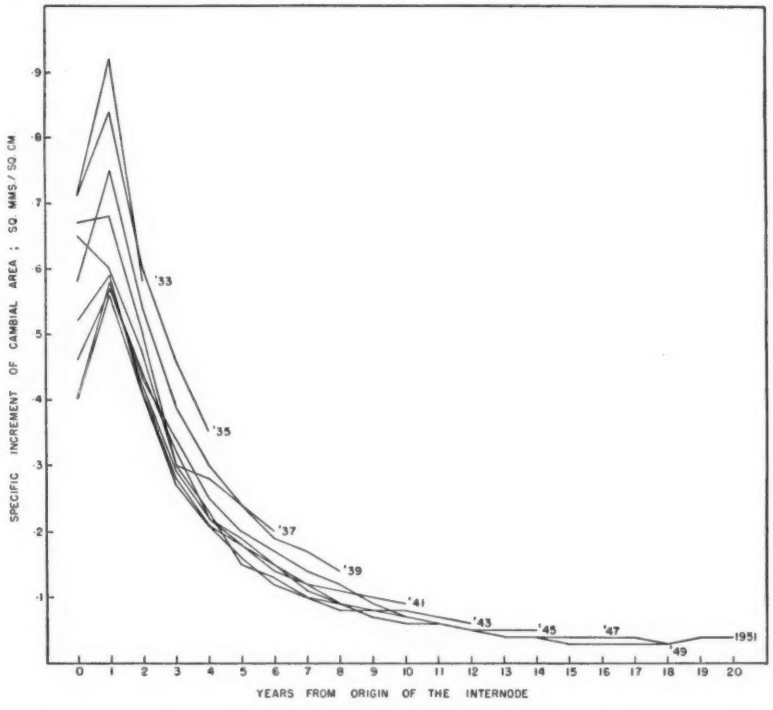

FIG. 10. Diagram of type 1 sequences of specific increment of cambial area plotted according to mode 2 so that the abscissal scale denotes the progressive age of the internodes during the growth period of the tree and the graphical origin represents the year of origin of every internode. Each line represents the specific increments of internodal cambia of progressive ages in a certain single year which is marked upon the diagram in such a way to identify the corresponding sequence. For the sake of clarity only alternate sequences are represented. Standard tree K52.1.

must be remarked with some emphasis because these are type 1 sequences, the longer of which pass down the axis from the apex of the tree or close to it, through the various gradations of the crown of foliage and beyond into a series of internodes in the lower zone of the tree that are not foliated but lie at progressively increasing distances from the foliage and its exported metabolic products. This succession of conditions is correlated with characteristic variations in specific wood volume increment (ring width) that we have denominated "pattern". The essence of the pattern is the development of a maximum in that portion of the tree's axis which runs through the upper part of the crown of foliage (13). Similar evidence of pattern is present in type 1 or type 2 sequences of increment of cambial area and increment of cambial circumference but not the slightest suggestion of pattern and hence of the effect of the intrinsic factor of foliar origin appears in the form of the type 1 sequences of *specific* cambial increment. On the contrary, the absence of anything resembling a maximum is sufficiently clear and the regularity of the hyperbolic form is sufficiently great to suggest that the internal factor of foliar origin, so influential in controlling the distribution of variation in specific wood volume increment, has minimal differential influence, or none, upon specific increment of cambial area.

Each sequence begins with high values and these decline with progress down the axis from internode to internode. The first terms of the sequences represent the specific increments of the cambia in the year of their origin in the tree's leading shoot of that year and the last terms represent the increments of the oldest, that is the earliest-formed cambia. The first terms of the type 1 sequences represent, accordingly, the increments of the younger (and smaller) internodal cambia while the latest terms are the increments of the older (and larger) cambia. In these circumstances, for reasons that have already been considered, the plotted data must automatically take the form of a decline with time and with cambial size. The only major deviation from perfect conformity to this rule in Fig. 10 is the relative smallness of the specific increment of the leading internode. The explanation of this lies in the severe time restriction to which cambial growth is subjected in its year of origin in any internode. The growth of the primary and intercalary meristems occupies the first six weeks of the season. This time ordinarily includes the best of the cambial growing conditions and in every internode secondary cambial growth cannot begin in the year of origin until late, after the cambium has been organized. For this reason the internodal secondary wood ring of the first year is always very narrow by contrast with the average of the succeeding rings. But because the wood ring is formed around a considerable pith the cambial areas, initial and final, are proportionately larger than ring width in the year of origin. The specific increment of cambial area in that year, though much larger than the average, is, accordingly, smaller than that of the succeeding term of the sequence. Thus arises the only exception appearing in the regularity of the data for this growth function the distributive conformation of which is so very different from the "pattern" for specific wood volume increment and must therefore be differently determined.

Sequences of type 2 exhibit their characteristics quite clearly in Fig. 9 and it has therefore not been thought necessary to record them in a special figure. Their conformation obviously resembles that of the first type sequence. The reason for this clearly is that the two types of sequences both involve the time scale of diameter but not of terminal growth. Therefore, when reading from early internodal growth increments to late we are, in both cases, reading from young (and small) to old (and big) internodal cambia. Accordingly, the automatic effect of increasing cambial size ensures that the geometrical form of the sequences of both types will be that of a decline of gradually diminishing slope along the cambial time scale. Thus, when the evidence of both types of sequence is considered, it is clear that the conformation of specific increment is similar whether the determinants are referred to changes in control which are formally described along the axis of the tree in any given year or along the cambial time scale at any given internode in the axis of the tree.

Third Type Sequences of Specific Increment and Their Conformation

The hyperbolic decline of specific increment of cambial area in the sequences of types 1 and 2 is not the only regularity manifest in the data. Close inspection of Fig. 10, comparing the sequences of early years (*ca.* 1933) with those of later years (*ca.* 1951), shows that entire sequences diminish in pitch from one year to the next. There is some irregularity, especially in the values for the cambium in the year of its origin, so that the lines of the plotted sequences intersect here and there. But in general, in any type 1 sequence of specific increment of cambial area, any point for a later year than the first is lower than the corresponding point of the previous year in the mode 2 plot. But each group of points taken at any given position on the axis of abscissae in the mode 2 plot of a type 1 sequence represents the terms of a type 3 sequence. There must therefore be a downward trend from early to late, or, if we plot the sequences with the apex of the tree at the origin, a conventionally upward trend, in the type 3 sequences. The trend is somewhat obscured by irregularity and by the narrow range of its values, but its effects are nevertheless distinguishable in Fig. 9 as well as Fig. 10.

The reality, extent, and distribution of the trend can most readily be made evident by plotting representative third type sequences as in Fig. 11 in which they appear in a plot of mode 2. The terms of the sequences there given correspond to rings that were formed 2, 3, 4, 5, 7, 9, 11, and 13 years from the origin of their respective internodes (i.e. numbers of the rings from the pith as shown in Fig. 9). The other sequences are omitted merely to prevent the figure from being obscured by too many lines. Fig. 11 presents the same relations for specific increment of cambial area that Fig. 16 of our earlier paper (13) gives for specific wood volume increment (ring width). The conformation of the sequences is manifest and the trend appears unmistakably in spite of the irregular fluctuations that they also exhibit. Fig. 11 additionally comprises a graph of internode length to which we shall refer presently.

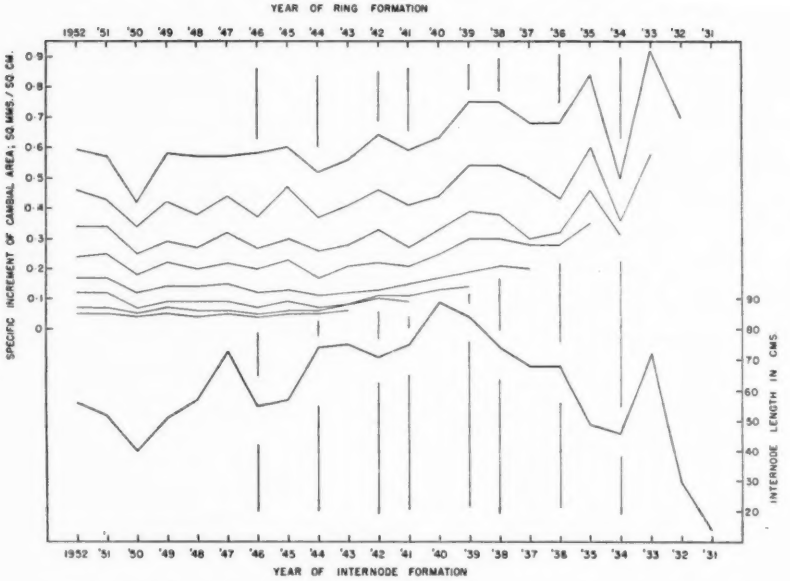

FIG. 11. Family of type 3 sequences of specific increment of cambial area plotted according to mode 2. They are thus aligned upon a common time scale of the years in which the rings were formed. For the sake of clarity certain of the sequences have been omitted from the diagram. Plotted below on the corresponding time scale are the lengths of the internodes laid down by terminal growth each year. Standard tree K52.1.

The range of the trend can be indicated more directly by referring to Table I in which are given the mean specific increments of cambial area for the earliest (basally situated) and latest (apically situated) five terms of the sequences plotted in Fig. 11. Manifestly the trend has a very much narrower range of values and proceeds with far less regularity than the corresponding trend in sequences of the first and second type (Fig. 10, Fig. 9). In the latter the range is a greater than 10-fold one and the regularity is almost diagrammatic (Fig. 10).

TABLE I

MEAN VALUES FOR THE EARLIEST AND THE LATEST FIVE SPECIFIC INCREMENTS OF CAMBIAL AREA TO BE FORMED IN THIRD TYPE SEQUENCES OF TREE K52.1

Designation of the sequence by its number from the pith*	Mean specific increments in sq. mm. per sq. cm.							
	2	3	4	5	7	9	11	13
Mean value of the five earliest-formed increments	0.69	0.48	0.35	0.30	0.19	0.11	0.078	0.05
Mean value of the five latest-formed increments	0.55	0.41	0.30	0.22	0.15	0.10	0.064	0.05

*Equivalent to designation by the age in years at which the internodal cambium formed the rings comprising the terms of each sequence.

Just as significant as the differences between the first and third type sequences in regularity and in range of numerical variation is the difference in their time relations. As we have seen and as Fig. 9 shows, sequences of the first and second type follow the time scale of diameter growth whereas it is clear that the time scale of type 3 sequences is that of terminal growth. Progression along the former scale entails progressive increase from term to term in both age and size of the internodal cambia; along the latter, progressive increases in the age of the terminal meristem and in its progressively increasing remoteness from the root system are involved. In our first paper (13, pp. 500–507) we dwelt exhaustively upon the distinctions between these two time scales which are brought about mainly by the fact that the secondary wood (cambial) growth increment is produced simply in a single year's growth cycle whereas terminal growth is a complex, compounded of bud formation and axial extension. Bud formation occurs in one year and axial extension in the year following. We considered the consequences of this distinction between the time scales of cambial and apical growth, developed the concept of the "precession of cambial growth", and from it developed a reconciliation of the variations occurring in the type 3 sequences of internodal wood volume increment (ring width) and those occurring in the growth activity of the apical meristem (internode length). We cannot recapitulate these many considerations here but will merely note that the two growth time scales are clearly very different in their functional connotations. The fact of this difference will be taken for granted in the present discussion.

Factors Controlling the Conformation of Type 3 Sequences of Specific Increment

We have already referred the hyperbolic decline of each separate type 1 sequence of specific increment (the "intrasequential trend") to the automatic effect of progressively increasing cambial size as the dominating but not necessarily the unique determinant. In type 3 sequences the intrasequential trend cannot be referred to the automatic factor because the terms of any given third type sequence are the increments of internodal cambia which are of equal age and approximately equal size. Third type sequences of mean cambial area for tree K52.1 are given in Fig. 6 and it is manifest from this aspect of the data that cambial area exhibits no tendency progressive with time to which the intrasequential trend of specific increment might be referred. The third type sequences of cambial area are, on the other hand, evidently influenced powerfully by the internode length parameter, the variation of which is in no wise progressive.

In type 1 sequences of specific increment the progressive intersequential trend is secondary to the intrasequential trend of the third type sequences. We have just concluded that the latter is independent of progressive changes in cambial size and we must therefore extend this conclusion to cover the intersequential trend in first type sequences. This, like the intrasequential trend of third type sequences is thus not determined by the automatic effect of progressive increase in cambial size. The intersequential trend of type 3

sequences, however, is secondary to the intrasequential trend of type 1 sequences and is therefore referable to the automatic factor as its dominating determinant.

A disproportionately great interest attaches to the intrasequential trend of the type 3 sequences of specific increment of cambial area. The reason is that the conformation of the trend, which is that of a gradual decline of diminishing slope, indicates the operation of systematic controls. Yet the conformation is incompatible with the mode of incidence of all recognizable, systematic controlling factors of extrinsic origin and also with the systematic controlling factor of foliar origin which we earlier found (13) to be the dominating factor determining the distribution of specific wood volume increment (ring width). The latter is the only intrinsic systematic factor of which we have as yet had recognizable evidence. It seems clear that a systematic factor, the effects of which have not hitherto been encountered in our analysis of growth, must now be recognized in action here. Some clue to its attributes should be deducible from a consideration of the intrasequential trend of the sequences of type 3.

The conformation of the trend necessitates that the factor should be progressive and that it should therefore give no evidence of maximum (or minimum) values at any intermediate point of time or of axial position. It is also clear from the small range of variation in the values of specific increment that the numerical variation of the factor must be small. Yet because the influence of the factor is exercised upon cambial increment its functional significance has an importance far beyond its numerical dimension. Wherever the regeneration and augmentation of the cambium is achieved — and that is everywhere throughout the internodal cambia — the factor controlling this function must be in effective operation. Thus it everywhere underlies the integral growth of the tree.

A consideration of the possible modes by which the controlling factor we seek to identify might originate and act suggests that it is associated with the regular progression in time of some attribute of a meristem. We have already seen that the meristem in question cannot be the cambium alone. The progression of the factor controlling the variation of specific increment in type 3 sequences takes place along the time scale of terminal growth and the apical meristem must therefore be involved. Accordingly, we must consider in the first instance an age series in the apical meristem, or the functional equivalent of such a series. It is evident that alternative forms of hypothesis are possible: Either the changes occurring progressively with time in the apical meristem induce corresponding secondary changes in the activity of the internodal cambia or the factor that brings about the changes in the apical meristem concurrently induces corresponding variation in the internodal cambia, but not secondarily through the apical meristem. In our present state of knowledge this might well prove to be an elusive distinction and the most useful hypothesis for the moment will evidently be one which comprehends both alternatives.

In our view it is very probable that the apical meristem of the main axis undergoes progressive changes of metabolic state with time and progressive vertical growth. Manifestly each successive annual increase in the length of the axis causes the apical meristem to become more remote from the source of its water supply, its mineral supply, and from the supply of organic factors of remotely proximal origin and especially those originating in the root system. This consideration alone might be deemed sufficient to justify adoption of the hypothesis that progressive changes are thus induced in the apical meristem without specifying whether the ultimate effect on cambial activity is achieved as a secondary consequence of the changing state of the apex or concurrently and in parallel with the apical progression. It is advisable to reiterate at this point that the cambial progression, whether secondary to the apical or parallel to it, could hardly be detected, except where the time scale is that of apical growth, because of its being masked in the first and second type sequences of specific increment, by the automatic effect of increasing cambial size. But however this may be, this form of the hypothesis is manifestly very general and lacking in highly desirable particularity as to the mechanism of the action. This disability can be overcome to some extent through invoking contemporary developments in the field of enzyme synthesis.

These developments stress the importance of "inductors", that is, of substances which constitute an essential part of an enzyme-forming system. Though the induction phenomenon was originally recognized under special circumstances and though the enzymes formed in response to the presence of the inductor were regarded, accordingly, as special products and denoted as "adaptive enzymes", it now appears that the phenomenon of induction is of much more general significance than was originally supposed. The view is gaining that much, perhaps even all, enzyme synthesis is inductive (12, 14, 16, 18, 21, 22, 23). We have only to suppose that amongst the organic factors of radical origin which are transported to the apex of the tree are one or more inductors of essential enzymes to convert the proposed hypothesis from an undesirably general to a more suitably particular form. Moreover this form is one which offers hope of future experimental verification.

In no other organic form are the developments in the inductive generation of enzymes more likely to prove enlightening than in the apical growing points of the roots and shoots of plants. According to this view, the stream of metabolites proceeding distally along the axis toward the apical meristem comprises the inductors of enzymes that are to form part of the complements characteristic of the succession of different growth phases and metabolic states that every growing point embodies. The structural relations of the apical meristems to the integral plant make it probable that long-continued terminal growth in a tree form such as *Pinus resinosa* would have a progressively restrictive effect upon the arrival at the terminal growing points of both the main and the lateral axes of certain inductor substances of remote origin. If some of these factors should be found to originate in the apical growing points of roots, as we may well expect, then evidently in such cases the total restriction would be augmented by the effect of the terminal growth of the root being

added to that of the growth of the stem axis, both forms of growth contributing to the progressive spatial separation of source and sink. In such circumstances, granted the general validity of the inductive principle in enzyme production, it is possible to propose that the synthesis of certain enzymes and therefore metabolic and growth activity at the apical meristems is affected correspondingly by progressive restriction of the supply of inductor substances to the stem apices. It would also be possible to hold that the effect upon the cambium could be equally well induced either concurrently in parallel with the apical effect or as a secondary consequence of the effect upon the apex.

Brown and Robinson (11), considering the problems of growth and enzyme generation at the apical growing point of the root, provide convincing evidence of the necessity of what they denote as "growth induction". The production of new cells by mitosis in the meristem is by itself insufficient to ensure their further growth and development. Newly-formed daughter cells are not equipped to proceed immediately with growth. An inductive process intervenes and only after induction can growth and development occur. The induction is biochemical and the inductive agency is evidently a substance or substances of metabolic origin. The normal mode of access of these factors to the terminal meristematic cells is by transport centrifugally along the axis of the root. None of the factors in question have as yet been identified but the earlier work of Brown, Robinson, and Johnson (10) on the germination of the seeds of *Striga hermonthica* and the effect of root exudates upon extension growth suggests very strongly that quite simple substances of saccharide constitution are capable of playing the part of growth inductors in appropriate instances. It is difficult to conceive of the induction of growth as something distinct from the induction of enzyme and we consider that it is unnecessary to conceive them as separate. The whole conception of the progressive pattern of growth and development propounded by Brown and Robinson can be understood in terms of inductive enzyme generation proceeding under the influence of a progressively modifying supply of inductor substances to the meristem, accompanied by continued protein turnover. It is in exactly these terms that we conceive the origin and mode of action of the factor which controls the intrasequential trend of the third type sequences of specific increment of cambial area. Since the factor is progressive with time it presents the appearance of an aging factor of the apical meristem but whether we are entitled to regard it as a true senescent process or even as an element of senescence should remain, certainly for the present and perhaps permanently, a suspended question.

One other matter remains to be considered before our discussion of the conformation of the third type sequences of specific increment of cambial area can be concluded and this has to do with the graph of internode length that appears in Fig. 11 and the variations exhibited by this graph as well as those appearing in the family of sequences of specific increment. As we have already pointed out, Fig. 11 corresponds exactly with Fig. 16 in our previous paper. The comparison that we are about to draw between the variations in

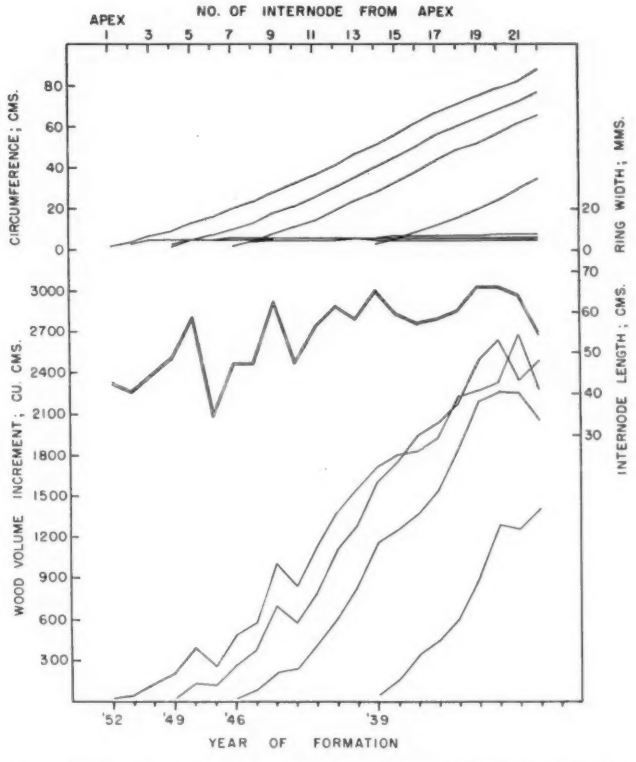

FIG. 12. Diagram of four type 1 sequences of internodal wood volume increment (below) and corresponding sequences of the cambial circumference, ring width, and internode length parameters. This figure corresponds exactly to FIG. 2 but data are for comparison tree K52.2.

internode length and in the type 3 sequences of specific increment of cambial area is also strictly analogous to that which will be found in subsection V.2, pp. 503–507 of our previous paper (13), where the comparison lay between the very same variations of internode length and those of the type 3 sequences of specific wood volume increment (ring width).

The first consideration is that the gross topography of the internode length graph on the one hand and that of the family of sequences on the other is different in the present Fig. 11 though the two were alike in Fig. 16 of the previous paper. The major topographic features of the sequences in former Fig. 16 were attributed to the influence of extrinsic factors of site and stand density. It follows, therefore, that the quite different major features of the sequences of present Fig. 11 must be given some other attribution. This has, of course, already been done. The major features in question constitute the intrasequential trend. The control of the trend has been discussed at length

in this subsection and has been attributed to a systematic, intrinsic factor correlative with an age series in the apical meristem. It is interesting that specific wood volume increment, the additive component of cambial growth activity, and specific increment of cambial area, the multiplicative component, should contrast so sharply and unmistakably in the dominating controls of their major features. It is equally interesting that the dominating control of the additive and numerically big component should be extrinsic in origin while that of the multiplicative and numerically small but physiologically fundamental component originates intrinsically.

The further consideration has to do with the minor topographical features, that is, the fluctuations of random incidence that are exhibited by both the graph for internode length and by the family of type 3 sequences of specific increment. In the corresponding comparison in respect of specific wood volume increment (ring width) made in our earlier paper (13) it was found that the minor fluctuations exhibited "precession of cambial growth" between 1934 and 1946 while at other times cambial growth and terminal growth fluctuated synchronously. The full discussion of this comparison cannot be repeated. It is recorded in the earlier paper (13, pp. 503–506) to which the reader is referred. Here we need only to draw attention to the fact that the minor fluctuations of specific increment of cambial area and of terminal growth again exhibit precession of cambial growth between 1934 and 1946 while at other times they vary synchronously. Manifestly therefore the controls of the minor random fluctuations of specific wood volume increment (ring width) are the same as those for specific increment of cambial area. The former were attributed to the influence of the unstable, annually variable factors of climatic origin, and this attribution must therefore now be extended to cover the latter measure of growth activity. It is, accordingly, clear that the factors of climatic origin which influence the empirical measure of growth activity in the apical meristem (internode length) affect likewise the measures both of additive growth activity and of multiplicative growth activity in the cambium.

IV. Results for Comparison Trees

1. Wood Volume Increment

Tree K52.2 was very similar to K52.1 except that whereas the latter was one of a plantation stand which had reached the stage of mutual suppression, the former was grown in the open. Both were trees of rapid growth and both were established on favorable sites. Fig. 2 shows the graphic data for wood volume increment and its geometrical parameters derived from K52.1, Fig. 12 for K52.2. Together with these we record in Fig. 13 the data for K47.5, a tree with a complex history (13), of slower growth and suffering from more severe suppression than K52.1, but like the latter, a forested tree.

Attention is drawn to differences of scale in the three figures. The scale of internode length, the center graph drawn in double line in each figure, is, in all, drawn to the same scale. The sequences of wood volume increment are in K47.5 fivefold the scale of K52.1, and in K52.2 these are drawn to one-third

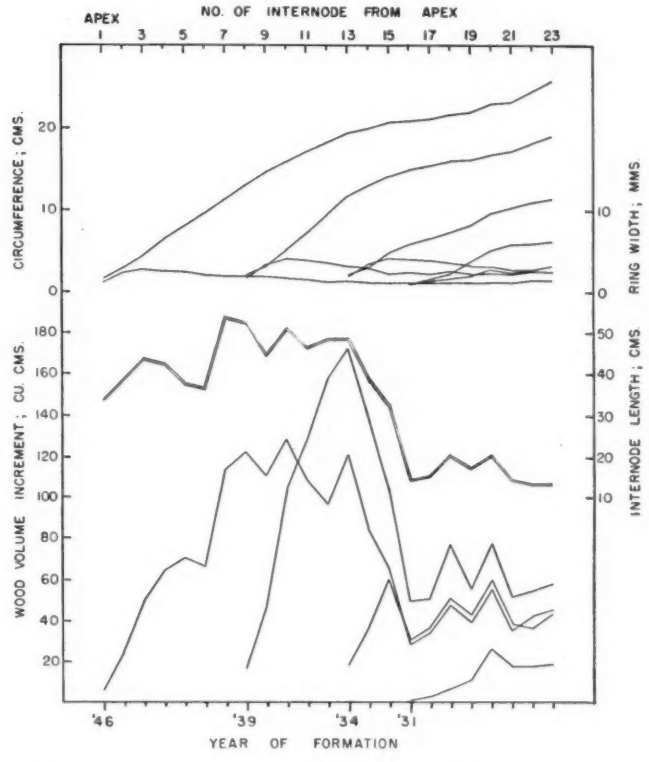

FIG. 13. Diagram of four type 1 sequences of internodal wood volume increment (below) and corresponding sequences of the cambial circumference, ring width, and internode length parameters. This figure corresponds exactly to FIGS. 2 and 12, but data are for comparison tree K47.5.

the scale of K52.1. In K47.5 the scale of the circumference parameter is twice that of K52.1 while in that of K52.2 the scale is one-half K52.1. The scale of ring width is in K47.5 twice that of K52.1 and four times that of K52.2.

It is clear that in both comparison trees the ring width parameter introduces little sequential variation into the data for wood volume increment. The dominant influence of the circumference parameter is visible in all parts of the volume increment sequences of tree K52.2 which nowhere are adjusted closely to the sequential variations of internode length, and the wood growth can be said to be cambium dominated. This point has already been considered in subsection III.1. By contrast, the internode length parameter dominates a greater part of the lower reaches of tree K47.5 than it does in tree K52.1. The factor of foliation and vigor is therefore markedly important and two contrasting comparison trees reveal in connection with wood volume increment and its parameters the differences from K52.1 that our consideration of wood

volume increment in the latter tree led us to expect. The open grown tree is "cambium-dominated", the severely suppressed tree "apical meristem-dominated" over a large proportion of their respective lengths where wood volume increment is the criterion of growth.

2. Mean Circumference

The type 1 sequences of cambial circumference, mode 2, for K52.2 and K47.5 are given in Figs. 14 and 15 respectively. The complete foliation of the former tree is marked by a considerable reduction in the range of differences between the various members of its family of sequences which lie together even more closely than do those of K52.1. Fig. 14 is plotted on an ordinate scale one-half that of Fig. 7 and Fig. 15 on twice that of Fig. 7. The mean slope of the sequence for the fully foliated tree K52.2 is actually greater than that for K52.1 and much greater than that for K47.5. Comparison of Figs. 2, 12, and 13 shows that the individual type 1 sequences of K52.2 show much less tendency to flatten off toward the top than do those of K52.1 and very much less than those of the severely suppressed tree K47.5. The bending over of the sequences corresponds in both trees that lacked foliage in the lower zone of the tree to the transition from active to dead branches equally in position and in degree.

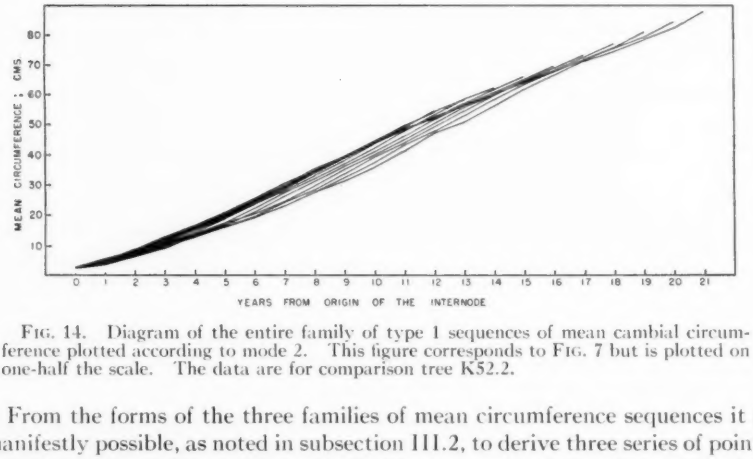

FIG. 14. Diagram of the entire family of type 1 sequences of mean cambial circumference plotted according to mode 2. This figure corresponds to FIG. 7 but is plotted on one-half the scale. The data are for comparison tree K52.2.

From the forms of the three families of mean circumference sequences it is manifestly possible, as noted in subsection III.2, to derive three series of points corresponding to the maximum (or limiting) amount of growth in girth that had been achieved in internodes of every age in the three trees. Plotted together on equal scales this would yield a diagram in which the amount of growth in girth could be contrasted internode by internode of corresponding age during the growth of the trees from the juvenile to the fruiting condition. Such a diagram consisting of graphs represented by points is given in Fig. 16. The ordinate values are plotted so as to compare the maximum amount of growth in girth achieved by internodes of corresponding age in all three trees for the first 20 years of determinable growth.

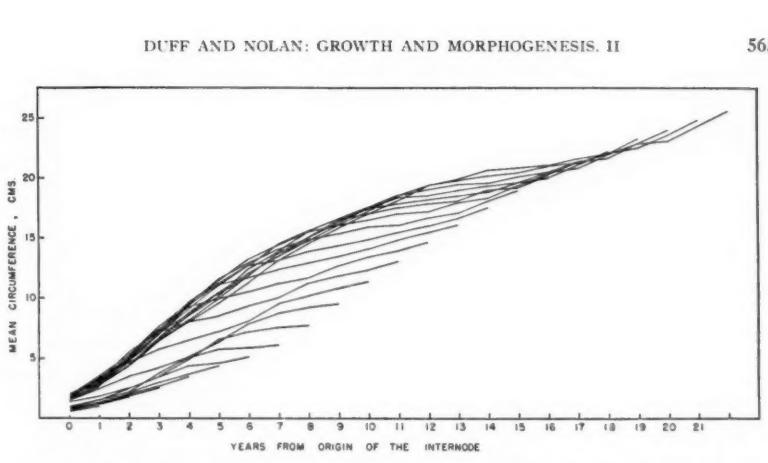

FIG. 15. Diagram of the entire family of type 1 sequences of mean cambial circumference plotted according to mode 2. This figure corresponds to FIGS. 7 and 9 but is on a scale twice that of FIG. 7 and four times that of FIG. 14. The data are for comparison tree K47.5.

It is evident that the open grown tree K52.2, which was otherwise favorably situated and made optimal growth throughout, nevertheless exhibits a slight falling away in the progression of maximum mean circumference in the later years of growth in the older internodes. The ruled straight line marks in the figure the conformation that would have resulted if internodal increase in girth had continued undiminished. The fact that the open grown tree did not maintain its maximum increase in the lower zone is of some interest. Our previous consideration of specific wood volume increment (ring width) in this tree (13) led us to the conclusion that the open grown tree of this species was subject to a certain amount of self shading. This had had a determinable effect upon specific increment of wood volume (ring width) and it is probable that the effect is also visible in the falling away of maximum mean cambial circumference in the same tree.

Tree K52.1, which grew in a favorable site but was one of a plantation stand which closed in upon one another at a fairly early age, kept pace with K52.2 during the early years of free and open growth undiminished by mutual suppression. But with the onset of suppression growth in girth fell off markedly in the later years of the older internodes. That is, it fell off in the lower zone of the tree where the foliage had been lost as the result of "natural pruning", and even in the lower middle zone of the tree which evidently suffered some inhibitory effects of the closing-in, though the foliage was not killed off.

Tree K47.5 grew under less favorable conditions than the other two, even at the beginning. Its curve of maximal mean cambial circumference exhibits a correspondingly lower slope from the first and the slope changes downward in the lower zone like that of K52.1, but to a greater degree, as the result of severe suppression.

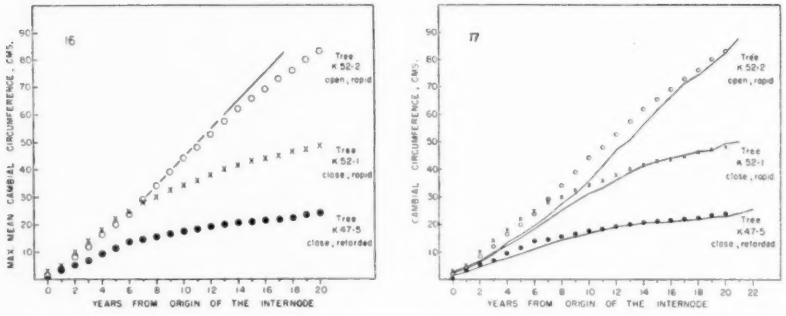
16
MAX MEAN CAMBIAL CIRCUMFERENCE, CMS.
YEARS FROM ORIGIN OF THE INTERNODE
Tree K 52-2 open, rapid
Tree K 52-1 close, rapid
Tree K 47-5 close, retarded
17
CAMBIAL CIRCUMFERENCE, CMS.
YEARS FROM ORIGIN OF THE INTERNODE
Tree K 52-2 open, rapid
Tree K 52-1 close, rapid
Tree K 47-5 close, retarded

FIG. 16. Diagram comparing the maximum mean cambial circumference achieved in internodes of every age in the three trees K52.1, K52.2, and K47.5.
FIG. 17. Diagram comparing maximum mean cambial circumference with the cambial circumference of the most recent first order sequence in trees K52.1, K52.2, and K47.5.

We may safely assume that the range of difference exhibited by the three trees represented in Fig. 16 is by no means the greatest that would be encountered among reasonably well-growing specimens of *P. resinosa* within this part of the species' range. A wider survey would undoubtedly enlarge the range of difference greatly. In all probability it would also disclose distinctions in the conformation of the graphs that result from such differences in the conditions of growth as liberation from suppression at various stages of development with corresponding effects upon cambial activity in different parts of the tree. The three instances before us here were in steadily developing conditions of open or closed growth at the time of sampling. They therefore represent instances of minimal complexity, as far as they go. We would expect to find this measure of growth very sensitive to the effects, both augmentative and inhibitory, of factors of every kind, intrinsic and extrinsic. We believe, accordingly, that a comprehensive study of growth in these terms would be repaid.

Fig. 17 has been inserted to demonstrate that though maximum, mean circumference may be the more nearly ideal basis of comparison between the cambial growths of such trees as these, nevertheless the most recent first order sequence of cambial circumference gives in all internodes a sufficiently close approximation to the maximum to serve the purposes of comparison except where the differences in question are small. Such sequences could be defined to a sufficiently close approximation without destruction of trees by annual diameter measurements taken at all internodes, or for practical purposes, at every n^{th} internode, the frequency being chosen in relation to the purpose in view. Provided the readings were made by a suitable method the error entailed in the fact that the external diameter is measured rather than the diameter at the cambium should not impair the value of the data for many comparative purposes. Prominent amongst the purposes for which a relatively simple and surveyable datum of this kind would be useful are the comparison and evaluation of site.

3. Specific Increment of Cambial Area

The last comparison to be made in this section is the one based on the sequences for specific increment of cambial area, the multiplicative component of cambial growth, which is equivalent physiologically and equal numerically to specific increment of cambial circumference. Figs. 18 and 19 present the type 1 sequences for trees K52.2 and K47.5 respectively and these are to be compared with the corresponding data for tree K52.1 which appear in Fig. 10. Again, only half the total number of sequences is represented in the figures and the choice of the sequences for years of odd number is arbitrary.

The regularity and uniformity in the members of the families of sequences is once more noticeable and particularly remarkable in the sequence for tree K52.2. There is a considerably greater range of variation in K47.5 than in K52.2 and a somewhat greater range than in the standard tree K52.1. The form of the individual sequences (the intrasequential trend) is, however, but slightly modified as between the three trees we are comparing. This strong similarity in intrasequential trend confirms our earlier conclusion, reached from a consideration of the first type sequences of specific increment of cambial area for tree K52.1 alone, that the intrinsic metabolic-nutritive factor which constitutes such an important control of specific wood volume increment (i.e. ring width), the additive component of cambial growth, has a minimal effect or none upon specific increment of cambial area, the multiplicative component. We have seen from the data for tree K52.1 that specific increment of cambial area is greatly influenced by the automatic effect of progressively increasing cambial size when this growth function is regarded in terms of sequences of the first or second type. But the function, as we have seen in III.6, is also influenced by a second intrinsic determinant which in sequences of types 1 and 2 but not in sequences of type 3, is masked by the automatic effect. The impressive similarity in the intrasequential trend of type 1 sequences of the three trees shows that the dominating determinant is the same in them all and we conclude that both comparison trees are affected by the two intrinsic determinants in generally similar manner to K52.1.

Though the differences in intersequential trend might be supposed to be of secondary magnitude and interest, they should be brought to account nevertheless. In the first place, the range of homologous differences between the type 1 sequences of K52.2 (the homologous intersequential differences) is smaller than that of tree K52.1. The differences for tree K47.5 have a greater range of variation than that for the standard tree K52.1. The magnitude of the homologous intersequential differences thus appears to be a direct correlative of growth activity in all parts of the tree. This in turn suggests that the comparatively close approximation of the K52.2 sequences to coincidence (Fig. 18) is an indication that in this very actively growing tree internodal cambial growth approached its limit year after year in all internodes of the tree. Considering the cellular basis of specific cambial growth this might well have been predicted for maximally growing trees. But no such relation appears in the data for tree K47.5, which was severely suppressed in respect of wood

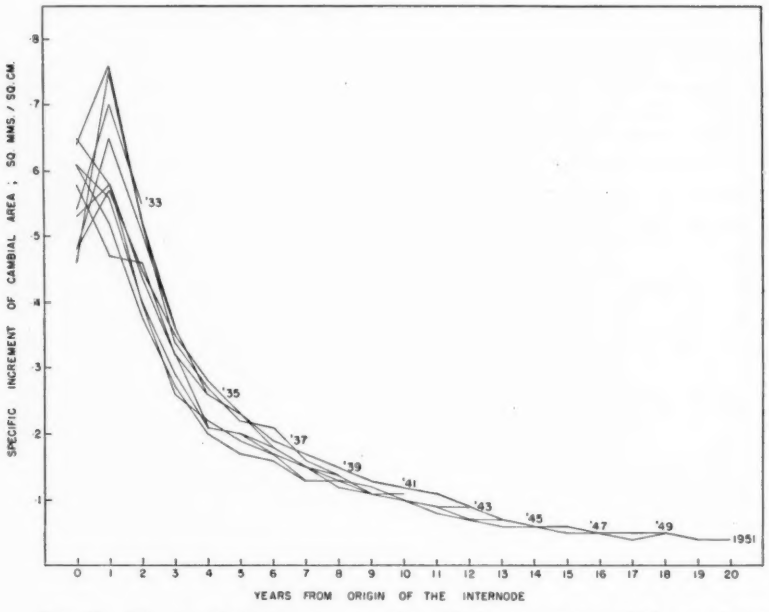

FIG. 18. Diagram of type 1 sequences of specific increment of cambial area plotted according to mode 2. This figure corresponds exactly to FIG. 10 but the data are for comparison tree K52.2.

production, and considerably more severely than K52.1 (13). Tree K47.5 manifests the least tendency to coincidence of its type 1 sequences of cambial growth and the homologous intersequential differences are relatively large. It is clear that net cambial growth activity was suppressed in K47.5 as well as wood growth and was suppressed to a corresponding degree, as must be expected since growth of wood and growth of cambium are exact correlatives owing to the geometry of the tree.

The third type sequences of K52.2 and K47.5 both show the downward intrasequential trend that was found in the corresponding sequences of K52.1 (Fig. 11, Table I). The range of the trend in the comparison trees may be indicated most readily by the data of Tables II and III, which correspond to Table I for tree K52.1 and with which they may be compared. It is manifest that the ranges of the No. 2 and No. 3 (2nd and 3rd year) sequences of K52.2 and K52.1 are indistinguishable and that the main difference between the trends in these two trees lies in the reduction of the downward trend to an indeterminably small value in the 4th year sequence of K52.2 whereas the trend persists on a determinable scale in K52.1 until the internodal cambia that produce the terms of the sequences were in their 6th–7th year. In tree K47.5 the range of the 2nd year sequence is not so great as it is in K52.1 but the 3rd year sequence corresponds closely and the downward trend persists in this severely suppressed tree throughout the body of collected data.

TABLE II

MEAN VALUES FOR THE EARLIEST AND LATEST FIVE SPECIFIC INCREMENTS OF CAMBIAL AREA TO BE FORMED IN THIRD TYPE SEQUENCES OF TREE K52.2

Designation of the sequence by its number from the pith*	Mean specific increments in sq. mm. per sq. cm.							
	2	3	4	5	7	9	11	13
Mean for five earliest-formed increments	0.68	0.49	0.32	0.25	0.19	0.15	0.11	0.08
Mean for five latest-formed increments	0.56	0.41	0.31	0.24	0.17	0.13	0.10	0.08

*Equivalent to designation by the age in years at which the internodal cambia formed the rings comprising the terms of each sequence.

TABLE III

MEAN VALUES FOR THE EARLIEST AND THE LATEST FIVE SPECIFIC INCREMENTS OF CAMBIAL AREA TO BE FORMED IN THIRD TYPE SEQUENCES OF TREE K47.5

Designation of the sequence by its number from the pith*	Mean specific increments in sq. mm. per sq. cm.							
	2	3	4	5	7	9	11	13
Mean for five earliest-formed increments	0.58	0.48	0.34	0.28	0.21	0.17	0.12	0.08
Mean for five latest-formed increments	0.57	0.40	0.27	0.20	0.12	0.07	0.05	0.04

*Equivalent to designation by the age in years at which the internodal cambia formed the rings comprising the terms of each sequence.

The intrasequential range of the third type sequences is by definition numerically equal to the homologous intersequential differences of the first type sequences in any tree. The intrasequential ranges of difference in the type 3 sequences characteristic of trees K52.2 and K47.5 are thus manifestly concordant with the conformation of the families of type 1 sequences for the same trees and confirm the conclusions regarding their determinants that we derived in the first instance from a consideration of the data for tree K52.1. The modifications that we observe in the type 3 sequences are such as would be expected to follow from trees showing widely different growth activities as a result primarily of differences in the degree of suppression resulting from the density of the stand and not from differences in the character of the critical intrinsic control.

Since the evidence of the comparison trees is confirmatory of the conclusions and consistent with the hypotheses advanced from the data for the standard tree we may consider the ground firm enough to sustain one further suggestion regarding the conditioning of cambial growth activity and its distribution in the tree. This is that the stream of organic substances migrating along the axis whether acropetally toward the growing point or basipetally away from it plays an important and perhaps a decisive part in determining the result of the competition between the potential cambial initials that arise by anticlinal

division. Success means retention as additional cambium cells; failure means ultimate maturation or elimination. The competition could hardly be conceived except in terms of access on the part of the competing cells to a factor or factors of a nutritive-metabolic order.

From the nature of wood growth, the nutritive-metabolic factor determining the activity of the cambium in this kind of growth must be held to be (or at least to comprise) a factor of large quantity supporting the synthesis of massive quantities of the organic components of wood. This factor is held to be of foliar origin because of the observed relation between the distribution of wood growth activity and that of the well-illuminated foliage of the tree (13). All its attributes thus concord with the view that the factor is of photosynthetic origin and could properly be denominated "photosynthate".

The nature of cambial growth is almost the converse of that of wood growth. Cambial growth depends upon no massive supply of "photosynthate". The mass annual increment of new cambial tissue in a complete annual increment is very minute by contrast with the corresponding increment of wood. The competing cellular products of periclinal division that finally maturate to xylem undoubtedly require a much larger supply of "photosynthate" to achieve their fate than do the "successful" competitors which remain meristematic. The critical factor or factors for cambial growth would appear to be factors of small quantity, contrasting in magnitude with "photosynthate".

FIG. 19. Diagram of type 1 sequences of specific increment of cambial area, plotted according to mode 2. This figure corresponds exactly to FIGS. 10 and 18 but the data are for comparison tree K47.5

It is therefore probable that they contrast also in mode of action. These circumstances invite the conclusion that the critical determinant which settles the fate of a potential cambial cell is a substance or substances of hormonal nature. We propose to identify them with the substances postulated in subsection III.6 as the intrinsic factor controlling the intrasequential trend in third type sequences of specific increment of cambial area.

The mechanism of the action of these factors upon the meristems was envisaged in III.6 as that of inductors. The same conception is equally applicable at this point, the essential requirement of which is a mechanism by which originally similar potential cambial cells become distinguished from one another and are caused to pass to quite different fates, including their retention as meristematic elements. If this view should prove to be valid in detail, including the premise that a positive differential growth or enzyme induction is exerted upon the cells that remain meristematic, the action must be exerted on the cambium by components of the stream of metabolites passing acropetally along the axis toward the apical meristem. This would establish the hypothesis that the cambial induction is achieved in parallel with apical induction and not as a secondary consequence of the apical effect. All this follows because the highest cambial growth activities occurred in the earliest formed rings of the earliest formed internodes and so were achieved when the tree was young and small. These rings came to lie in the lower and inner part of the trunk as the tree grew older. The distally situated rings exhibit progressively lower activities (Fig. 9). Thus the diminishing axial gradient of cambial growth activity, only observable when unmasked by the automatic factor as in type 3 sequences, is in its spatial aspect acropetal even as it progresses along the time scale of apical growth in its temporal aspect (Figs. 9 and 11). If, however, the control should turn out in the end to be that of an inhibitory action on surviving cambial cells or that of an inductive process in one of the "unsuccessful" cell competitors rather than in the cells that remain meristematic, it is more probable that the factor would arrive at the reaction center in the stream of metabolites passing basipetally and not acropetally along the tree's axis. This same alternative in different guise was confronted in subsection III.6. There we concluded that until definitive evidence is available the most valuable form of hypothesis is one which comprehends both alternative possibilities.

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THE BIO-ELECTRIC POTENTIALS OF PLANTS AND THEIR FUNCTIONAL SIGNIFICANCE

I. AN ELECTROKINETIC THEORY OF TRANSPORT[1]

D. S. FENSOM[2]

Abstract

The background of bio-potentials in relation to transport is briefly reviewed and the chief difficulties lying in the way of an electrical theory of transport are pointed out. A re-examination of electro-osmosis and streaming potentials in xylem and phloem vessels suggests that the bio-electrical forces are sufficiently large in magnitude to cause transport and that they certainly operate in a direction to assist it. The productions of the potentials through metabolic activity and membrane diffusion is discussed.

Introduction

Although circulation of fluids in plants has been known and studied for many years, it is well recognized by plant physiologists that the mechanisms so far suggested are not completely adequate to account for this circulation. The theories currently accepted have their limitations.

For instance, soil water can presumably enter a root by molecular cohesion and by osmosis (1, 14, 16) but how does this water get out of the concentrated cell sap into the lumina of the xylem where the tracheal sap is often reported to be dilute (23)? Do the semipermeable membranes change their nature from one side of a cell to another so that water can only pass in one direction? Once the mechanism of pumping water quickly into the vessels of the xylem has been established, the upward transport, whether by vapor streams (45) by molecular cohesion or just by so-called root pressure, seems to be fairly easy to understand. Meanwhile "diffusion pressure deficit" is the term usually given to explain the anomaly (23).

On the other hand the descent of sap in the sieve tubes presents quite a difficult problem. The known dimensions of the sieve tubes and the frequent barriers of sieve plates, not to mention the probable protoplasm in the tubes, provide an enormous resistance to the mass flow of sap (12, 13).

In the face of these difficulties, the theory of electro-osmosis seems to offer a possible mechanism to assist the bridging of the problems. It is well known that liquids can be caused to move along capillary tubes under the influence of an electromotive force applied across the ends of the tube. The volume of flow is proportional to the difference in potential, and the flow is directed

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Contribution from Ridley College, St. Catharines, and the Glendon Hall Laboratory and Garden, Department of Botany, University of Toronto, Toronto, Ontario. Paper read to the Symposium on the Physiology of Trees, Sixth Research Conference on Plant Physiology, Ottawa, October 31, 1955. Acknowledgment is made of grants from the National Research Council of Canada and the Ontario Research Foundation.
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towards the terminal with the same polarity as the tube walls. Cellulose and protoplasm have a negative potential compared with water, and hence dilute solutions will move towards the negative pole. In general, the interior of a root is electrically negative both to the root surface and to soil water. The apex of a healthy tree is usually positive to the base (43).

History of Bio-potentials in Relation to Plant Transport

In 1878, Kunkel (24, 25) suggested that water transport was the cause of plant electricity. Then in 1892 Haake (18) supposed that plant bio-potentials were due to respiration or assimilation effects but could find no correlation between transpiration and current (amperes). However, in the 1930's the theory received quite an impetus from the work of Lund (26–34), Marsh (38, 39), and Stamm (54) in the United States, from Marinesco (37) in France, and from Heyl (19) in Germany. Yet in spite of the correct polarity in most trees and good correlations between potentials and oxygen gradients, large difficulties arose. In 1944, Lundegardh (35) published a calculation of the potential required to raise water in the xylem vessels to a height of 100 cm. This was of the order of 150,000 mv. and hence he concluded that "electro-endosmosis in the accepted meaning of the term will certainly not be a factor in bleeding". This opinion has often been quoted.

Another difficulty lies in the fact that published reports do not show any obvious correlation between transpiration and potentials. For instance, Burr's work (6, 8, 9) on maple trees at Yale, while showing seasonal and daily cycles, does not disclose a simple connection to transpiration and even exhibits polarities which would seem to oppose it at certain seasons. On the other hand there are reports that potentials in plants are affected by insect attack (42), by poisons (53, 55), by synthetic growth regulators (11, 15), by tropisms (4, 50, 51, 52), and by an inherent electrical field around each cell (7, 10). Finally, since bio-potentials may be accounted for in terms of membrane permeability (5, 41, 55) as well as by the oxidation–reduction (46, 47) systems of metabolism, there has been a quite reasonable tendency to attribute potentials to transport rather than the reverse. Before the theory of electro-osmotic transport can be considered seriously there will have to be not only theoretical grounds for thinking it possible, but also experimental evidence of correlation between flow of fluids and bio-potentials.

Yet in spite of these weighty difficulties, a number of physiologists seem to remain open-minded on the subject. For instance, Bennett-Clark (2, 3) suggests electro-osmosis into plant vacuoles, Lundegårdh (35, 36) has his anion respiration in root tips, Höber (20, 21) has pictured membranes with pores of different size which will in effect be electro-osmotic in action. Crafts (13) and Kramer (23) both appear to be well aware of the possibility of this phenomenon, though they seem to prefer other mechanisms. Hence it would seem to be worthwhile to re-examine electrokinetic phenomena in narrow tubes as they might apply in plants.

Electrokinetic Equations

The two expressions commonly used to describe electrokinetic movement of liquids in narrow passages are the well-known Helmholtz–Smoluchowski (40) equations, that is, for electro-osmosis, where an applied e.m.f. produces a flow of liquid:

(1) $$f = \frac{q\,\epsilon\,\zeta}{4\,\pi\,\eta}\,\frac{E}{l}$$ (for significance of symbols, see Appendix),

and streaming potential, where there is a pressure causing flow of liquid which produces a difference in electrical potential between the ends of the flowing column,

(2) $$V = \frac{\epsilon\,\zeta\,p}{4\,\pi\,\eta\,l\,k}.$$

Transport in the Xylem

Suppose these two equations are applied to the tracheal tubes of the xylem where the capillaries have a reasonably uniform cross section, then the volume rate-of-flow becomes equal to the product of the cross section of a capillary and the velocity rate-of-flow within it. Hence

$$f = qv$$

and converting from electrostatic to ordinary units

(3) $$v = \frac{\epsilon\,\zeta}{4\,\pi\,\eta\,(300)^2}\,\frac{E}{l}.$$

In the xylem vessels

ϵ is approximately 80,

η is approximately 0.01 poise,

ζ may be taken as about 0.013 (the figure is Stamm's (54) but is for cellulose–water systems. Recent work suggests that where protoplasm is involved this figure may rise perhaps 10 times).

In any one system at a given temperature these figures will not change very much. Hence

(4) $$v = 0.92\times10^{-4}\,\frac{E}{l}.$$

To get a flow velocity of 10 cm. per hour, an e.m.f. of some 4500 *volts* would be required along 150 cm. of tracheal tube (with the upper electrode negative). The potential differences found in trees seldom exceed 50 mv. along 150 cm. of stem. Hence this is the type of calculation which leads Lundegårdh to say that electro-osmosis in xylem vessels is not a force which contributes significantly towards bleeding (35).

It might be noted that in circular tubes when dealing with the velocity of flow, the cross-section of the tube drops out of the calculation. This is because the flow is a "plug type" (40) originating from the electro-static nature of the charges on the tube walls. The neutral cellulose walls seem to attract water molecules by hydrogen bonding, and they are left with an apparent negative charge. It is assumed that this charge is distributed evenly on the surface, also that the potential gradient is constant throughout the capillary, and that the capillary is large compared to the space charge on the molecules.

Similarly, for a streaming potential in capillaries of uniform bore, one may apply the Poiseuille equation

$$p = \frac{8 f l \eta}{r^4},$$

where $f = q v = \pi \frac{d^2}{4} v$;

then

$$V = \frac{8 \epsilon \zeta v}{9 \times 10^{11} k \pi d^2} \text{ in ordinary units.} \tag{5}$$

Hence, using the same values as before,

$$V = 2.9 \times 10^{-12} \frac{v}{k d^2} \cdot \tag{6}$$

When the rate of flow is around 10 cm. per hour and tracheal diameters are about 20 μ (Preston (44)) as in conifers, and k is 2×10^{-6} ℧ then the streaming potential

V is approximately 1 mv.

However, if k drops as low as 2×10^{-7} ℧ (Atkins (1)) and the rates of flow become high, say 100 cm./hour, the streaming potential rises to quite significant figures (with the upper electrode positive).

Again the potential is electrostatic in origin, but the flow causing it is streamline, not plug. It may be noted that streaming potentials in continuous xylem tubes will be significant only if transpiration rates are high, if liquid conductivity is very low (i.e. few ions present in the water), or if the tubes are small in diameter. These conditions may exist in the stems of trees towards the middle and end of summer, but at the base of the tracheae (in the root) and in newly formed tracheal tubes near the cambium these conditions do exist at any time and yet such appreciable electrical drags have not been reported.

On the other hand, if constrictions or membranes exist across xylem vessels as apparently is the case when these vessels are new, the potential gradients would be proportional to the resistance and significant gradients would exist, not along capillaries but across the barriers (particularly membranes). The holes through these barriers would now be so small that streaming potentials might be expected to be very large with the upper pole positive. Yet in healthy roots the inside is always negative to the tip, and in the stem before the leaves come out the upper electrode may be found negative to the base. Here again the application of the classical equations of capillary flow is not encouraging and experimental measurements have in the past appeared to oppose the physical theory.

However, more encouragement is encountered when the assumption is made that the observed potentials are metabolically maintained. For if potential gradients can be applied across these barriers by metabolic processes, then the barriers would act as electro-osmotic pumps to move water and small

ions. In equation (4) assuming that E is 100 mv. (an average potential across root interior to exterior) distributed chiefly across 10 barriers, each 1 μ thick, then where

$$v = 0.92\times10^{-4}\,\frac{E}{l}, \tag{4}$$

v becomes about 36 cm. per hour, with the correct polarity to that observed!

This barrier type calculation, while it cannot be taken as precise with the values for the parameters which are at present available, at least shows that the direction and the order of magnitude are correct for such a phenomenon to occur.

Transport in the Phloem

When the sieve tubes of the phloem are considered it becomes apparent that the observed potential differences (E) are not at all of the right order of magnitude to move the sap downward at the observed rates if the tubes are considered to be of uniform bore, free from sieve plates. But again, metabolically maintained potential differences across the barriers may be invoked. The potential gradients must operate largely across the plates themselves, and this would have the effect of making the operative voltage gradients (E/l) very much greater.

Let us substitute for the sieve tube a hypothetical capillary of uniform bore large enough to carry sap at the same velocity as in the original tube, but of length "l" equal to the combined thicknesses of all the sieve plates in an equal section of sieve tube. According to Schumacher (49) tube units 1 cm. long have barriers about 5 μ thick at the end. In 150 cm. of stem there would then be 750×10^{-4} cm. of barrier. Using these values along with a fairly high figure for potential measured along 150 cm. of stem, equation (4) becomes

$$v = 0.92\times10^{-4}\,\frac{E}{l} = 0.92\times10^{-4} \times \frac{0.050}{750\times10^{-4}} \text{ cm. per sec.},$$

or v is about 0.2 cm. per hour.

This flow rate is low, but is the correct order of magnitude and direction to that observed. (If the much higher values for ζ between water and live protoplasm be taken, the figure for flow will increase in proportion.)

If the flow in the sieve tubes is electro-osmotic in nature, it should be of a "plug" type across the sieve plates. Moreover, one would then expect large ions or charged molecules to move at somewhat different speeds from the surrounding sap owing to the electric fields near the plates. There is some evidence that both of these phenomena occur (12, 47).

On the other hand, if the flow is not electro-osmotic in origin, then the sap must be forced down the phloem by some pressure applied elsewhere in the plant, such as a very high osmotic or "diffusion pressure" gradient somewhere. This would produce streaming potentials across the barriers. Assuming the same zeta potentials as before, and tubes of bore equivalent to the holes in the sieve plates (maximum 2 μ), then for a velocity of flow of 2 cm. per hour,

$$V = 2.9\times10^{-12}\,\frac{v}{kd^2}\ ; \tag{6}$$

V is about 200 mv. with the lower electrode *positive*. This has never been reported, though small reversed potentials have been noted for instance when some constriction occurs in or around the sieve tubes.

Origin of Membrane Potentials

If electro-osmosis is a motivating factor for transport, there must be a continual source of potential gradient across the sieve plates of the phloem, the base membranes of xylem tracheal tubes, and any other place where this mechanism occurs. There are two very probable sources.

When transpiration rates are high (which tend to occur at times when the tracheal tubes will be of smaller diameter than in the spring), the streaming potentials produced in the xylem would seem to be appreciable. These potentials may possibly leak back into the phloem and increase the potentials across the sieve plates. The polarity would certainly be correct. This in effect would mean that some energy of transpiration will tend to assist sap transport downward.

The other source is probably much more important. The electrokinetic potential across a membrane separating two solutions may be found by an expression (36) as follows:

$$P = \frac{RT}{nF} \log_e \frac{(H^+)_2}{(H^+)_1},$$

where $(H^+)_1$ is the activity of hydrogen ion at side 1 and $(H^+)_2$ is the activity of hydrogen ion at side 2. The potential then depends on the relative concentration of anions and cations and the rates of diffusability of each across the barriers.

The structure of cellulose as revealed by Preston's studies (44) with the electron microscope suggests that the dry thickness of cellulose micelles is about 40 Å while the intermicellar thickness is about 100 Å. When wet, these distances might be reversed, but with protein coatings the gaps between the micelles will probably be much smaller, though ubiquitous. These gaps will almost certainly be permeable to hydrogen (hydronium) ions, but probably impermeable to large ions, particularly hydrated anions (such as bicarbonate) or organic acids. Hence any respiration of sugars on one side of a barrier which would lead to the formation of H^+ and HCO_3^- will leave that side of the barrier more negative, for the H^+ can diffuse away through the protoplasmic membrane and the cellulose. With the known negative zeta potentials of protoplasm or cellulose as Höber (20, 21) says, there will tend to be a continual diffusion of protons upwards across sieve plates and a continual slight increase in negativity from cell to cell downwards. Hence potentials across the sieve plate barriers will tend to operate electro-osmotically wherever there are large openings.

The ion production in each cell will be regulated by metabolic processes in the tube cells, in the companion cells, and in any meristematic tissue. Hydrogen ion diffusion and adsorption may be postulated in turn as regulated by the various metabolic processes and enzyme activities at or near the membranes.

Before leaving the subject of the origin of potentials it might be well to confront the difficulty inherent in assumptions about metabolism in the xylem. Except for currently developing elements just inside the cambium, the xylem elements appear to be relatively inert metabolically (17). It seems improbable that metabolism in the rays and wood parenchyma alone is sufficiently active to support the potentials observed. Hence if this theory is valid, the active pumping of fluids should be found to be associated also with the energy transformations of elements near the cambium and intercalary meristems and with elements near a root tip or a stem growing-point. Other xylem transport (such as transpirational pull) could, from this point of view, be associated with maturated woody tissue and incidentally would produce streaming potentials (upper terminal positive).

There is no corresponding problem in the phloem where the average metabolic activity is relatively high. The data of Goodwin and Goddard for the phloem of red maple and black ash (17) show that the activity of the phloem of woody species is comparable to that of leaves and other of the more highly metabolic tissues of plants, and appears, in general, to be exceeded only by tissues in the extension phase of growth.

It is suggested that this principle could also be applied to the movement of water from living, respiring cells in the root to dead xylem vessels provided there exists a mechanism for removing positive charges on the tracheal side. This may quite possibly exist.

Discussion

With the help of the Helmholtz equations of electro-osmosis and streaming potential it is possible to build up a theory by which the natural bio-potentials of plants may assist transport, and the inverse. It is, of course, well recognized that equations designed for tubes of macroscopic size may not necessarily apply when transferred to microscopic holes in sieve tubes or membranes. None the less, it is suggested that the theoretical picture produced is so consistent that the ideas should be considered most seriously and for this reason they are here described.

As far as is known, the theoretical objections raised to the idea of electro-osmotic transport can be met. There remain two important questions to be answered before the theory could be generally accepted: Can externally applied electrical potentials alter transport flow? Does experimental evidence exist to support the theory?

Although there have been some claims that applied potentials have altered transport (37), these are suspect, since conflicting reports exist (22, 48). However, according to the theory outlined herein, any e.m.f. applied externally along a stem would leak around the tissues and would oppose the flow of one stream if it assisted the flow of another. Moreover, the applied e.m.f. would not operate principally at the barriers but would act with a low potential gradient throughout many cells and tissues. This would tend to oppose some metabolic processes if it did not actually kill the cells.

The experimental evidence which exists is widely scattered and it is quite possible that the theory might be inconsistent with some of it. At the moment no experimental evidence is known which is seriously in opposition, while a number of experiments not here described suggest direct support to correlations between bio-potentials and transport. It is hoped to report upon these studies in a future paper. Meanwhile it is thought that the ideas summarized in this theory should be published for critical consideration.

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Appendix

Significance of Symbols

f = volume of electro-osmotic flow in cc. per sec.
q = cross section area of capillary, sq. cm.
E = applied electromotive force, e.s.u.
l = length of capillary, cm.
V = streaming potential, e.s.u.
p = pressure difference between ends of capillary, dynes per sq. cm.
k = specific conductivity of liquid in e.s.u.
ϵ = dielectric constant of the liquid
η = viscosity of the liquid in poises
ζ = zeta potential, e.s.u. between freely moving liquid and the walls of the capillaries
v = velocity rate of flow in cm. per sec.
r = radius of capillary in cm.
$mv.$ = millivolt
μ = micron
Å = Ångstrom units
P = potential difference across a membrane
R = gas law constant
T = absolute temperature
F = number of coulombs transferred
n = number of equivalents involved
$\mho$ = reciprocal ohm

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THE INHERITANCE OF COUMARIN IN SWEET CLOVER[1]

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Abstract

The inheritance of coumarin in white blossom sweet clover was determined from a study of crosses involving plants of the variety Arctic (containing free coumarin), Pioneer (containing bound coumarin), and coumarin-deficient selections. Both a fluorometric and a colorimeter test were used to detect the presence and type of coumarin.

Among high-coumarin plants, variation in the amount of coumarin was due to non-heritable causes or genetic factors not detected in this study. The cross of free-coumarin × bound-coumarin plants established that the bound-coumarin character was inherited as a simple recessive. The gene symbol *b* is proposed for this character. In the crosses of high-coumarin × coumarin-deficient plants the F_1 progeny were more or less intermediate in coumarin content and the subsequent F_2 established that coumarin production is governed by one partially dominant gene. The symbol *Cu* is proposed for this gene. The gene *B*, responsible for the free-coumarin production, expresses itself only in the presence of *Cu* and is undetectable in a coumarin-deficient plant homozygous for *cu*. Some atypical unexplained F_2 ratios are reported.

Introduction

Coumarin is an organic compound present in the plants of most species of sweet clover. It has a bitter, stinging taste and a vanilla-like odor. In addition to rendering plants of this crop relatively unpalatable to herbivorous animals, the anticoagulant dicumarol is evolved from coumarin when certain conditions prevail in haying or ensiling sweet clover (15, 16, 23). Therefore, plant breeders have attempted to develop coumarin-deficient varieties (4, 12, 14, 18, 19).

The colorimetric procedure for coumarin determination (2, 3) was used at the Forage Crops Laboratory to breed a presumably low-coumarin variety named Pioneer (18). Subsequently, the fluorometric method of analysis revealed that Pioneer had a high-coumarin content (12). The coumarin present in this variety was described by Slatensek and Washburn (12) as 'bound' coumarin because in non-hydrolyzed material it was not extractable with ethanol. In contrast, the ethanol extractable coumarin found in the vast majority of plants of *Melilotus alba* was described by Slatensek and Washburn as 'free' coumarin. More recently, strains have been developed at the Forage Crops Laboratory which are coumarin-deficient on the basis of any known test for coumarin.

A study was undertaken to determine the inheritance of the three classes of coumarin described above. The results of this study are presented herein.

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Literature Review

Using the colorimetric procedure for coumarin determination (2, 3, 17), Stevenson and White (18) showed that the low-coumarin character resulted from a single recessive gene. This explanation was confirmed by Horner and White (6), who used material and analysis procedures similar to those employed by Stevenson and White. Horner and White found no evidence of linkage between coumarin content and the Alpha habit of growth or white sepal color.

Rinke (9), using a modification of the colorimetric test (10), found the coumarin content of the F_1 to be similar to that of the low-coumarin parent, and 77.3% of the F_2 were classified as low-coumarin. He concluded that high-coumarin content was inherited as a simple recessive. The level of coumarin in the low-coumarin parent material used by Rinke was considerably higher than the low-coumarin material used by Stevenson and White (18) and Horner and White (6).

Smith (13, 14) crossed *M. dentata* with *M. alba* and propagated the chlorophyll-deficient hybrids by grafting the seedlings onto plants of *M. officinalis*. By backcrossing the hybrid to *M. alba*, some seed was obtained. From an analysis of the backcross progenies Smith (14) concluded that two main genes for reduced coumarin content had been obtained from *M. dentata*. One of these genes was recessive to high content, and the other displayed no dominance.

By embryo culture technique Webster (20) successfully crossed high-coumarin *M. officinalis* with low-coumarin *M. alba*. Based on the analysis of the F_2 population by the short fluorometric method he suggested a single major recessive gene for low-coumarin content.

Materials and Methods

In this study three classes of parental material were used: namely, coumarin-deficient, bound-coumarin, and free-coumarin. This classification is based on the coumarin reaction obtained in both the colorimetric and the fluorometric tests. In the coumarin-deficient class the plant material reacts negatively whereas in the free-coumarin class it reacts positively to both tests. In the bound-coumarin class a negative reaction is obtained with the colorimetric test and a positive reaction with the fluorometric test. Both the bound- and free-coumarin classes contain coumarin but the reaction of the coumarin to the test procedures is different in these two classes of material.

The Arctic variety was the source of the free-coumarin class of parental material. Twenty plants of this variety were selfed and from each a selfed progeny of three plants was grown and tested by the photofluorometric procedure. Wide variations in coumarin content were found both within and between progenies. Four of the strains were chosen as they represented fairly distinct differences in coumarin content and displayed limited variation among plants in the strains. The coumarin content of the plants in each of the four strains is given in Table I. Within each strain one of the three plants was

selected to represent the strain, and these four plants served as the free-coumarin parents. These plants are designated as Arctic-1 to Arctic-4 in this paper.

The Pioneer variety was the source of the bound-coumarin parental material. A small population of plants of this variety was grown and tested for coumarin by the colorimetric procedure. One plant was chosen as the parent and is designated as Pioneer-1 in this paper.

TABLE I

TOTAL COUMARIN CONTENT, EXPRESSED AS PERCENTAGE GREEN WEIGHT, OF THREE PLANTS OF EACH OF FOUR STRAINS OF THE ARCTIC VARIETY

	% coumarin in strain			
Plant	Arctic-1	Arctic-2	Arctic-3	Arctic-4
1	1.00	0.69	0.54	0.34
2	0.90	0.76	0.45	0.39
3	1.00	0.67	0.58	0.33
Average	0.97	0.71	0.52	0.35

The coumarin-deficient strains were derived from stock of (*M. dentata* × *M. alba*) × *M. alba* parentage, seed of which was supplied by Dr. W. K. Smith, University of Wisconsin, Madison, Wisconsin. At the Forage Crops Laboratory this stock was backcrossed to varieties and strains of *M. alba* for one or more generations. From this backcrossing program a number of coumarin-deficient strains were developed. In the present study a small population of each of a few of these strains was grown and tested for coumarin. One coumarin-deficient plant was selected from each of seven strains and used in the crossing program. Two of the seven coumarin-deficient plants were of the Alpha type, the growth form of which has been shown to be conditioned by a simple recessive gene (1, 8). The seven coumarin-deficient plants are designated A to G in this paper.

Crossing and Selfing Programs

Crossing and selfing were performed in the greenhouse where bees were completely excluded. Flowering was induced in this biennial material by providing a day length of 18 hours or more beginning about a month after planting. Flowering began about 4 to 6 weeks after supplemental light was provided. This method has been used for several years at the Forage Crops Laboratory, and a similar method has recently been described by Wiggans (24).

Each of the four selected Arctic plants was used as the pollen parent in crosses with each of the seven selected coumarin-deficient plants. Likewise Pioneer-1 was crossed with each of the coumarin-deficient parents. The Arctic plants were crossed with Pioneer-1, using the former as the female parent. The various coumarin-deficient plants were intercrossed in all possible combinations. In crosses involving coumarin-deficient plants with

Arctic and Pioneer plants the crosses were made in the direction specified above to permit detection of selfs. In the cross of Arctic × Pioneer, selfs were detected by the absence of segregation in the F_2.

Emasculation was performed on young fresh flowers by a suction method similar to that described by Kirk (7) except that a motor-driven pump supplied the negative pressure. Pollination was accomplished by applying to the stigma pollen collected on the slightly moistened broad end of a tooth-pick.

All plants used in the crossing program were also selfed. Selfing was accomplished by rolling the racemes between the thumb and forefinger. After manipulating the flowers of a plant the operator thoroughly washed and dried his hands before proceeding to the next plant.

Methods of Sampling Leaf Material

All of the plants used in this study were grown and sampled for coumarin in the greenhouse. The F_1 and selfed progenies were grown simultaneously. Plantings of F_1 and selfed progenies were repeated when the F_2 was grown. Leaves were collected from various classes of material at the early flowering stage when coumarin production was at a maximum (4, 5, 11, 17, 19, 21).

The sample for the colorimetric test consisted of one newly opened fresh leaf taken from the tip of a well developed branch. As an aid to uniformity, leaves sampled were those on which the middle leaflet was about $\frac{3}{8}$ in. × $\frac{1}{4}$ in. The selected leaf was immediately placed in a test tube containing 2 ml. of 95% ethanol. The stoppered test tube was left standing 24 hours before the test was continued.

For analysis with the photofluorometer, a sample of 100 mg. of fresh leaf material was taken. The fresh green leaves were selected from the meristematic region of the main stem and side branches, at or near the top of the plant. As a rule of thumb, leaves chosen for the sample were those on which the center leaflet had not fully opened. Preliminary studies had shown that the coumarin content of such leaves was as high as or higher than that of any other part of the plant. Immediately after it was weighed, the 100 mg. sample was placed in a test tube containing 15 ml. of 2.5 *N* sodium hydroxide, stoppered tightly, and stored in a refrigerator at 0° C. until time of analysis. Periodically 1-g. samples of fresh leaves were taken for moisture determinations.

The samples for the short fluorometric method consisted of two fresh young leaves obtained from the tip of the main stem. These leaves were immediately placed in about 10 ml. of 2.5 *N* sodium hydroxide, stoppered, and stored in a dark room at about 20° C. until time of analysis.

Methods of Coumarin Analysis

The colorimetric test was used to distinguish between the presence of bound and free coumarin. A rapid microchemical qualitative procedure outlined by Smith* was used. It consists of a modification of the colorimetric procedure

*Smith, W. K. A short colorimetric procedure for determination of coumarin. University of Wisconsin unpublished correspondence.

described originally by Clayton (2). At completion of the chemical procedure the samples are usually classified as either containing free coumarin or not containing free coumarin (bound-coumarin class).

For rapid determination of the presence or absence of total coumarin, the fluorometric method described by White *et al.* (22) and modified by Goplen *et al.* (5) was used. The coumarin content was scored usually on a scale of 1 to 5 where 1 is the coumarin-deficient condition.

A photofluorometric analysis was used to secure quantitative measurement of total coumarin. The procedure used in the present study was essentially the same as that described by Slatensek and Washburn (12) and subsequently revised by Slatensek (11). A Coleman photofluorometer, model 12B, equipped with B-1 and PC-1 filters, was used. Immediately prior to the reading of the samples with this equipment, the cuvettes containing the samples were irradiated with an ultraviolet light for 10 minutes. The meter values for the unknown samples were converted to percentage coumarin by reference to a standard curve. The standard curve was prepared from meter readings of standards prepared by addition of known amounts of coumarin to coumarin-deficient leaves.

Experimental Results and Discussion

Crosses were made between coumarin-deficient plants and Arctic plants representing different levels of coumarin. The crosses were made and the F_1 and selfed progenies of the parents were grown simultaneously in a greenhouse and coumarin determined quantitatively by using a Coleman photofluorometer. The data obtained from the selfed progenies are summarized in Table II. Among the high-coumarin material it is evident that the mean and the range of coumarin content of the different selfed progenies were essentially similar although the parent plants carried from 1.00 to 0.34% coumarin. The coumarin content of the progenies of coumarin-deficient plants was distinctly different from the progenies of high-coumarin plants. Evidence of a trace of coumarin was found in one or more plants of five of the seven strains but the majority of the plants were void of coumarin.

Each of the high-coumarin plants listed in Table II was crossed with each of the coumarin-deficient plants listed in that table. The data on coumarin content of the F_1 of these crosses are summarized in Table III. These data show that all F_1 progenies displayed essentially the same average and the same range of coumarin content.

The results presented in Tables II and III establish that the variation in coumarin content among high-coumarin plants is due to non-heritable or undetected heritable variation. In view of these results, the variation in level of coumarin among high-coumarin plants was disregarded in further genetic analyses.

In a comparison of Tables II and III it is seen that the average coumarin content of the F_1 was approximately one-half that of the selfs of the high-coumarin parent. The respective ranges of coumarin content of these two

TABLE II

COUMARIN CONTENT OF PARENTAL PLANTS AND SELFED PROGENIES EXPRESSED AS PERCENTAGE OF GREEN WEIGHT

Parent plants		Selfed progenies		
			Coumarin content in %	
Designation of plant	Coumarin content in %	No. of plants tested	Range	Mean
1. Free-coumarin strains				
Arctic-1	1.00	16	0.39–0.76	0.56 ± 0.123
Arctic-2	0.70	22	0.24–0.63	0.47 ± 0.144
Arctic-3	0.45	26	0.40–0.85	0.54 ± 0.141
Arctic-4	0.34	15	0.19–0.50	0.37 ± 0.087
2. Bound-coumarin strain				
Pioneer-1	0.49	6	0.66–1.05	0.88 ± 0.149
3. Coumarin-deficient strains				
A	0.00	10	0.00–0.03	0.008
B	0.00	9	0.00–0.01	0.001
C	0.00	10	0.00–0.04	0.004
D	0.00	10	0.00–0.00	0.000
E	0.00	8	0.00–0.04	0.005
F	0.00	6	0.00–0.00	0.000
G	0.00	5	0.00–0.01	0.002

TABLE III

FREQUENCY DISTRIBUTION FOR TOTAL COUMARIN CONTENT (EXPRESSED AS % GREEN WEIGHT) OF THE F_1 PROGENIES OF SEVEN COUMARIN-DEFICIENT PLANTS EACH CROSSED WITH FIVE HIGH-COUMARIN PLANTS (AS MALES) REPRESENTING DIFFERENT LEVELS OF COUMARIN CONTENT

	No. of F_1 plants with coumarin percentage of:							
Designation and coumarin content of male parent	.05 .09	.10 .14	.15 .19	.20 .24	.25 .29	.30 .34	.35 .39	Average coumarin content of F_1
Arctic-1 (1.00%)	—	2	17	17	12	11	3	0.25
Arctic-2 (0.70%)	—	16	14	19	11	—	—	0.20
Arctic-3 (0.45%)	4	13	17	13	4	5	—	0.18
Arctic-4 (0.34%)	8	9	17	8	2	5	3	0.18
Pioneer-1 (0.44%)	13	17	6	5	5	1	—	0.15

groups of material overlapped considerably, however. Thus it would be very difficult or impossible to distinguish between the heterozygous and the homozygous coumarin-containing plants in the F_2 on the basis of total coumarin content. For this reason and because of the saving in time and effort it was decided not to use the photofluorometric analysis. Two rapid semiquantitative procedures were employed for the coumarin analyses of the large F_2 population and F_1 and selfed progenies. Thus each plant was tested by two methods. The fluorometric method determined the presence or absence of coumarin and the colorimetric procedure the presence or absence of bound coumarin.

Cross of Arctic (Free-coumarin) × Pioneer (Bound-coumarin)

The selfed progenies of the four Arctic plants and of the Pioneer-1 plant contained respectively free coumarin and bound coumarin. No deviations were observed and thus the parent plants were considered homozygous for the genes governing the two types of coumarin. Eighty-two F_1 plants of this cross were tested and all gave a very strong test for free coumarin. In the F_2, 91 plants possessed free coumarin and 28 plants bound coumarin only, giving a good fit to a 3:1 ratio ($P = .95$–$.50$). As the fluorometric test revealed that all plants contained coumarin, it is evident that only the gene governing free coumarin vs. bound coumarin was segregating.

For the locus concerned with the development of free coumarin, the designation *b* will be used in this paper. Thus the genotype of the Arctic parents is *BB* and for the Pioneer parent is *bb*.

These results are identical with those obtained in previous studies at the Forage Crops Laboratory (6, 18). Rinke (9), however, concluded that the high-coumarin character was inherited as a simple recessive.

Crosses of Coumarin-deficient × Pioneer-1

The selfed progeny of Pioneer-1 contained bound coumarin only. The selfed progenies of the two coumarin-deficient plants C and E were practically void of coumarin (Table II).

From the cross of Plant C × Pioneer-1 all of the F_1 population contained only bound-coumarin. As shown in Table IV, the F_2 segregated for bound- and coumarin-deficient plants and gave a good fit to a 3:1 ratio.

TABLE IV

SEGREGATION FOR COUMARIN CONTENT IN F_2 OF TWO COUMARIN-DEFICIENT PLANTS × PIONEER

Coumarin-deficient parent	No. of F_2 plants containing:			χ^2 value for indicated genetic ratio	P value
	Free coumarin	Bound coumarin	No coumarin		
C	—	132	37	0.870 (3:1)	.50–.20
E	85	29	28	2.125 (9:3:4)	.50–.20

Crossing plant E with Pioneer-1 resulted in an F_1 which contained free-coumarin whereas only bound-coumarin was expected. Furthermore, as shown by Table IV, the F_2 segregated for free-coumarin, bound-coumarin, and coumarin-deficient plants in a ratio of 9:3:4. This establishes that two independent genes were involved, one of which was at the *b* locus which controls free- vs. bound-coumarin. The other locus, designated *cu*, controls the presence or absence of coumarin. In the homozygous recessive, *cucu*, the *B* gene is not expressed. On this basis the genotype of plant E is *BBcucu* and that of Pioneer is *bbCuCu*. The F_2 segregation pattern is shown in Table V.

TABLE V

GENOTYPIC CONSTITUTION OF COUMARIN-DEFICIENT PLANT E AND PIONEER-1 AND OF THE F_1 AND F_2 OF CROSSES BETWEEN THEM

Material	Assumed genotype and frequency of occurrence	Coumarin status
Plant E	*BBcucu*	Deficient
Pioneer-1	*bbCuCu*	Bound
F_1	*BbCucu*	Free
F_2	9 *B—Cu—*	Free
	3 *bbCu—*	Bound
	3 *B—cucu*	Deficient
	1 *bbcucu*	Deficient

As shown above when coumarin-deficient plant C was crossed with Pioneer-1, an F_2 ratio of three bound-coumarin plants to one coumarin-deficient plant was obtained. Therefore the genetic constitution of plant C is *bbcucu* and only the *cu* gene was segregating in this cross.

Referring back to the Arctic × Pioneer-1 crosses, now it may be deduced that the genotype of Arctic is *BBCuCu*. This interpretation would account for the observed ratio of three free-coumarin plants to one bound-coumarin plant.

Crosses of Coumarin-Deficient Plants × Arctic

From the cross of coumarin-deficient plant C × Arctic 77, F_1 plants were tested and all found to contain free coumarin. As shown by Table VI the F_2 provided a good fit to a 9:3:4 ratio. The genotype of plant C had previously been determined to be *bbcucu*, and the genotype of Arctic to be *BBCuCu*. Therefore the genotype and coumarin status of the F_1 and the F_2 are identical with that shown for these generations in Table V. The data verify this interpretation.

Crossing coumarin-deficient plant E with Arctic resulted in F_1 plants containing free coumarin. As shown in Table VI no plants containing bound coumarin were found in the F_2. Thus the absence of bound-coumarin segregates in the population under consideration, and verifies that the genotype of plant E was *BB*, a conclusion also arrived at from the plant E × Pioneer-1 cross.

TABLE VI

SEGREGATION FOR COUMARIN CONTENT IN F_2 OF THREE COUMARIN-DEFICIENT PLANTS × ARCTIC

Coumarin-deficient plant	No. of F_2 plants containing: Free coumarin	Bound coumarin	No coumarin	χ^2 value for indicated genetic ratio	P value
C	288	77	127	2.108 (9:3:4)	.50–.20
E	419	—	50	— (8.4:1)	—
G	146	—	17	— (8.6:1)	—

For the entire population of E × Arctic, however, the ratio of free-coumarin to coumarin-deficient plants was 8.4: 1. Although, as shown in Table VII, each of the F_2 families of this cross contained more than 100 plants, the ratios ranged from 4.8: 1 to 11.6: 1. The entire population of the cross of G × Arctic also gave a ratio of 8.6: 1. These over-all ratios indicated a possibility of two additional complementary genes that gave coumarin in the absence of *Cu*. If such were the case, the segregation for coumarin vs. no coumarin would be 57: 7.

TABLE VII

SEGREGATION FOR COUMARIN IN F_2 OF COUMARIN-DEFICIENT PLANTS E AND G WITH FOUR ARCTIC PLANTS

Cross	No. of F_2 plants containing: Free coumarin	Bound coumarin	No coumarin	Observed ratio
E × Arctic-1	102	—	15	6.8: 1
E × Arctic-2	117	—	10	11.7: 1
E × Arctic-3	96	—	20	4.8: 1
E × Arctic-4	104	—	9	11.6: 1
G × Arctic-1	104	—	11	9.5: 1
G × Arctic-3	42	—	6	7: 1

In order to determine whether complementary gene action of the above type is operative, the coumarin-deficient progeny of the F_2 of the cross E × Arctic were used in an intercrossing program. Of the 54 plants of this type, 52 were used in this part of the study. Ten of these 52 plants were picked at random and diallel crosses were made. These same 10 plants were also all crossed with the other 42 plants. They were also crossed with 10 coumarin-deficient plants of the F_2 progeny of plant C × Arctic. All crossing was done by hand in the greenhouse, using the suction pump for emasculation.

From the seed obtained, up to 10 seedlings were grown from each of the crosses. Of the diallel group 406 plants were grown and 1195 plants of the other groups were grown. All plants were tested for coumarin by the rapid fluorometric procedure, and all were coumarin-deficient, indicating that the deviation of the F_2 ratio of the cross E × Arctic from a 3: 1 ratio was not due to complementary gene action.

General Discussion

This study has established that two genes are concerned with inheritance of coumarin in *M. alba*. The gene designated as *b* determines the type of coumarin present (free or bound) in a coumarin-containing plant, while the gene symbolized as *cu* governs the production of coumarin. In the absence of *Cu* the *b* gene has no apparent effect.

On the basis of the data presented the genotypes of the parent are as follows:

PARENT	COUMARIN STATUS	GENOTYPES
Arctic	Free-coumarin	*BBCuCu*
Pioneer-1	Bound-coumarin	*bbCuCu*
Plant C	Coumarin-deficient	*bbcucu*
Plant E	Coumarin-deficient	*BBcucu*
Plant G	Coumarin-deficient	*BBcucu*

Crosses among Arctic, Pioneer-1, and plant C may be readily interpreted according to classical Mendelian ratios. Arctic crossed with plants E and G did not fit any of the expected ratios. The ratios for coumarin vs. coumarin-deficient of the families of these crosses varied from 4.8:1 to 11.7:1, the over-all ratio being approximately 8:1. Although all these families are statistically homogeneous the wide range of ratios indicates that plants E and G react differently when crossed with different plants of the same Arctic strain. In the cross of Pioneer-1 × plant E a 4.1:1 ratio was observed. While the lack of coumarin-deficient types is not as pronounced as in the cross with Arctic, nevertheless the *P* value for a fit to a 3:1 ratio of coumarin vs. no coumarin is only .10–.05. This result is very similar to one family of Arctic × plant E that had nearly the same total number of plants. It may be of some significance that plants E and G are homozygous dominant for the bound-coumarin locus and also react to give a preponderance of coumarin-containing plants.

All the coumarin-deficient plants trace back to the highly incompatible cross of *M. dentata* × *M. alba*. Although the coumarin-deficient parents are 10 generations removed from this interspecific cross there may be a residual imbalance in some plants which is responsible for these abnormal segregations. Classical Mendelian ratios depend on the assumptions of random segregation of factors, and absence of any differential viability of male and female gametes or differential survival zygotes. Non-validity of any of these phenomena would obviously obscure a simple Mendelian interpretation.

In the section on Methods and Materials it was pointed out that two of the coumarin-deficient parent plants were of the Alpha type. It was hoped to investigate possible linkage relationships between the gene for Alpha habit and genes for coumarin. Under the prevailing greenhouse conditions, however, it was impossible to positively identify the Alpha type of growth.

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COMPARATIVE STUDIES OF CORYNE SARCOIDES (JACQ.) TUL. AND TWO SPECIES OF WOOD-DESTROYING FUNGI[1]

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Abstract

A study of the cultural behavior of *Coryne sarcoides* (Jacq.) Tul. and two wood-destroying fungi, *Polyporus tomentosus* Fr. and *Coniophora puteana* (Schum. ex Fr.) Karst., shows varied production of cellulase, polyphenol oxidase, and lignin-oxidizing enzymes. All three species were capable of producing cellulases and polyphenol oxidases, but only *P. tomentosus*, a white-rot fungus, was able to oxidize lignin. On the basis of laboratory tests, *C. sarcoides* does not appear capable of causing decay, but its presence in the heartwood may affect the progress of decay by antagonism to wood-destroying fungi. The antagonism of one isolate of *C. sarcoides* reduced the decay caused by *C. puteana* and *P. tomentosus* by from 50 to 75% of that obtained in controls.

Introduction

Because of its frequent occurrence with heart rot and its possible importance in the heart-rot problem, an ascomycetous fungus identified as *Coryne sarcoides* (Jacq.) Tul.[3] has been under investigation in this laboratory for the past few years. The distribution of this fungus together with some notes on its occurrence in the heartwood of decayed and healthy trees have already been reported (3, 4, 6). The present paper deals with certain aspects of the physiology of this fungus in an attempt to elucidate its role in the development of decay in forest trees. Several isolates of *C. sarcoides* from various localities and hosts have been examined and compared with isolates of wood-destroying fungi to study the occurrence of wood-degrading enzymes, the ability of the fungus to cause decay, and the effect of its presence on the decaying activity of associated heart-rot fungi.

Materials and Methods

Twelve isolates representing British and Canadian strains of *Coryne sarcoides*, four isolates of *Polyporus tomentosus*,[4] a white-rot fungus, and three isolates of *Coniophora puteana*, a brown-rot fungus, were used in this investigation. Data about the cultures are given in Table I.

Several media were used for the growth of the cultures examined.

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[2]Forest Biology Laboratory, Calgary, Alberta.
[3]Identified by Miss Doreen Wells of the Botany and Plant Pathology Division, Ottawa, Ontario.
[4]The name *Polyporus tomentosus* Fr. may be regarded as synonymous with the name *P. circinatus* var. *dualis* Peck, which has been used previously in papers originating from this Division.

TABLE I

SOURCE OF CULTURES

Fungus	Culture No.	Host	Locality
Coniophora puteana (Schum. ex Fr.) Karst.	A263	*Picea glauca*	Nordegg, Alberta
	C4	*Picea mariana*	County Champlain, P.Q.*
	C5	*Picea glauca*	Wasagaming, Manitoba*
Polyporus tomentosus Fr.	A361	*Picea engelmannii*	Burmis, Alberta
	A79	*Picea glauca*	Eagle Creek, Alberta
	A20	*Picea glauca*	Fawcett Lake, Alberta†
	339	*Picea engelmannii*	Burmis, Alberta
Coryne sarcoides (Jacq.) Tul.	T157	*Picea glauca*	Bow River Forest, Alberta
	L2C	*Picea glauca*	Lac la Biche, Alberta
	A56	*Picea glauca*	Fawcett Lake, Alberta†
	A29	*Picea glauca*	Fawcett Lake, Alberta†
	A110	*Picea glauca*	Otter Creek, Alberta†
	C103	*Pinus contorta* var. *latifolia*	Strachan, Alberta
	C52	*Pinus banksiana*	Ontario, Canada*
	BX1	*Fagus sylvatica*	Middlesex, England
	BG2	*Fagus sylvatica*	Middlesex, England
	BA3	*Fagus sylvatica*	Middlesex, England
	A11	Fruiting body	Windsor Great Park, England
	FC1	*Pinus contorta*	?, England

*Obtained from the Mycological Herbarium, Department of Agriculture, Ottawa, Ont.
†Collected by members of the Forest Biology Laboratory, Saskatoon, Sask.

1. (*a*) Two per cent malt extract agar (*b*) Gallic or tannic acid agar: 1.5% malt extract; 2.0% agar; tannic or gallic acid at a final concentration of 0.5%

2. Medium A

Casein hydrolyzate (vitamin free)	120 mg./liter
KH_2PO_4	1.5 g./liter
$MgSO_4$	0.5 g./liter
Glucose	400 mg./liter
Thiamine HCl	1 mg./liter
Yeast extract	300 mg./liter

Cellulose, in the form of filter paper (No. 1 Whatman) macerated in a blender for 2 minutes, was added at the rate of 5.0 g./liter.

3. Medium B

As in Medium A, but in place of cellulose, the glucose was increased to 20.0 g./liter. The yeast extract was reduced to 100 mg./liter.

For the production of cellulase the cultures were grown on 40 ml. of Medium A in 12 oz. medicine flats at 22.5° C. for 17 days. The contents of each flat were filtered through muslin to remove the larger particles, then the remaining

liquid was pressed from the mycelial matter in the muslin. The filtrate was centrifuged at 10,000 r.p.m. for 10 minutes to make it cell-free, and stored at −17° C. until required for the assay. Cellulose for the substrate was prepared by macerating filter papers in a blender cup with 500 ml. of distilled water for 2 minutes and this was then left standing overnight at 4° C. to extract impurities. After the supernatant liquid was poured off, the cellulose was dried and shredded. Cellulolytic activity was measured using the following conditions: 0.1 g. (to give a 2% final concentration in the mixture) of the shredded cellulose was weighed and mixed with 1.25 ml. of distilled water in 25 ml. bottles until homogeneous. To this, 1.25 ml. of Sorensen's sodium citrate buffer[5] and 2.50 ml. of filtrate were added and the mixture again stirred. The bottles were then sealed and incubated in a constant temperature bath at 27° C. for 17 hours, and the mixture kept agitated. The assay was made by determining the glucose in a sample of the mixture before and after incubation by the copper–iodometric method (8, 9).

Oxidase activity was tested qualitatively on gallic and tannic acid and lignin substrates. The production of polyphenol oxidase was tested on tannic and gallic acid agar (Medium 1 (*b*)) in Petri dishes according to the method used by Davidson, Campbell, and Blaisdell (2) for the differentiation of white- and brown-rot fungi. The ability of the fungi to oxidize lignin was tested according to a method recently developed by the author (5), which depends on the oxidative browning of the residual lignin in extracted wood meal. The tests were made by growing the fungi on 1 g. aliquots of the wood meal in 100-ml. flasks containing 5 ml. of Medium B. The presence or absence of a brown diffusion zone in the wood meal was recorded at the end of 10 days.

The decaying abilities of the fungi were tested by calculating the loss in weight of the test-blocks of wood, which had been incubated with the fungus for 3 months. Two different procedures for these tests were followed:

(1) Decay at different moisture levels was tested on blocks of wood measuring $1 \times 1 \times \frac{3}{4}$ in. which were sawn in sequence in series of nine from air-dry samples of spruce heartwood. Two holes were drilled halfway through each block to take the water amendment and a plug of wood inoculum. The moisture content of an end block of each series was determined, then, assuming this was applicable to the sister blocks, the oven-dry weight for each block was calculated. The blocks were surface sterilized with propylene oxide gas by the method of Hansen and Snyder (7), placed in sterile widemouth screw-top jars (5.5 cm.×5.5 cm.), and the moisture content adjusted to the appropriate level by adding sterile distilled water from a hypodermic syringe to the blocks. Inoculations were made by placing a plug of wood inoculum into the cavity provided for this purpose.

(2) Each isolate was tested on three blocks of wood similar in size to those described above, but without the cavity. These were placed one on top of

[5]The optimum pH for cellulolytic activity was found to be 4.4 with carboxymethyl cellulose as substrate and with *C. puteana* as the test fungus.

the other in screw-top jars to which had been added 3 g. of spruce wood meal enriched with Badcock's accelerator (1). The lowermost block rested on glass rods which were placed on the wood meal. Water was added to the blocks to give a moisture content of approximately 70% of their dry weight. The jar assemblies were sterilized by steam for $\frac{3}{4}$ hour, then three plugs of agar from an actively growing plate culture of the appropriate fungus were placed on top of the enriched wood meal. The decay cultures in both tests were kept in a saturated atmosphere for 3 months.

Experimental Results

Cellulolytic Activity

A comparison was made of the cellulolytic activity of the enzymes produced by the fungi when grown on Medium A (Table II). With the exception of the filtrate from culture L2C of *C. sarcoides*, which had no action on the cellulose, all the cultures produced cellulase(s) when grown on this medium. The filtrates of *C. sarcoides* were in most cases as active, and those of the British isolates more active, than the filtrates of the wood-destroying fungi. The British isolates showed a much greater cellulolytic activity than the Canadian isolates, a characteristic which might be interesting to compare with pigment formation, as this phenomenon appears to be weaker in cultures of the British isolates.

TABLE II

CELLULOLYTIC ACTIVITY OF FILTRATES FROM CULTURES OF *Coryne sarcoides*, *Polyporus tomentosus*, AND *Coniophora puteana*

Species	Isolate	Activity of filtrate*
Coryne sarcoides (British isolates)	BX1	.140
	BA3	.116
	BG2	.101
Average		*.119*
Coryne sarcoides (Canadian isolates)	L2C	.000†
	C103	.070
	A29	.065
	A110	.022
	C52	.061
	T157	.023
	A56	.028
Average		*.045*
Polyporus tomentosus	A361	.056
	A79	.041
	A339	.060
	A20	.048
Average		*.051*
Coniophora puteana	A263	.029
	C5	.107
	C4	.083
Average		*.073*

*Activity in terms of reducing sugars as mg. glucose per ml. of reaction mixture, incubated 17 hours at 27° C. Each value is based on two determinations.

†This value does not figure in the averages.

TABLE III

REACTION AND GROWTH OF *Coryne sarcoides*, *Polyporus tomentosus*, AND *Coniophora puteana* ON TANNIC, GALLIC ACID, AND LIGNIN SUBSTRATES

		Oxidation activity*					
		Tannic acid		Gallic acid		Lignin	
Species	Isolate	Reaction	Growth	Reaction	Growth	Reaction	Growth
Coryne sarcoides	BA3	−	Yes	−	Yes	−	Yes
	BX1	++++	Yes	++++	Yes(tr.)	−	Yes
	BG2	++++	Yes	−	Yes	−	Yes
	L2C	−	Yes(tr.)	−	No	−	Yes
	C103	−	No	−	Yes	−	Yes
	A39	−	No	−	Yes	−	Yes
	A110	+++	Yes	−	Yes(tr.)	−	Yes(tr.)
	C52	−	No	−	Yes(tr.)	−	Yes
	T157	++++	Yes	−	Yes(tr.)	−	Yes
	A86	−	Yes	−	Yes	−	Yes
Polyporus tomentosus	A361	+++	No	++++	Yes(tr.)	+ (18)	Yes
	A79	++++	Yes(tr.)	++++	Yes(tr.)	+ (18)	Yes
	A339	++++	Yes	++++	Yes(tr.)	+ (22)	Yes
	A20	++++	Yes(tr.)	++++	No	+ (13)	Yes
Coniophora puteana	A263	+	Yes(tr.)	−	Yes	−	Yes
	C5	+	Yes	−	Yes	−	Yes
	C4	−	Yes	++	Yes	−	Yes

*The measurement of growth on tannic and gallic acid media was in terms of colony diameters of Petri dish cultures at the end of 7 days; growth on lignin was in terms of the pH change of the wood meal after 10 days. The basis for the reaction ratings on tannic and gallic acid was according to the scheme of Davidson *et al.* (2), while the reaction on lignin was determined by the presence or absence of a brown diffusion zone in wood-meal cultures (5). The diameter (mm.) of the reaction zone is shown in parentheses.

Polyphenol Oxidase Activity

The polyphenol oxidase activities of the fungi are shown in Table III. Cultures of each of the three species gave positive phenolase reactions but, as might be expected, only the four isolates of *P. tomentosus*, a white-rot fungus, gave consistently positive results on the two substrates. Positive reactions were obtained for culture C4 of *C. puteana* on gallic acid and for cultures of A263 and C5 on tannic acid. The action on these substrates by the brown-rot fungus was surprising because production extracellularly of polyphenol oxidase is usually associated with fungi of the white-rot type. Of the 10 cultures of *C. sarcoides* tested, only four, BX1, A110, BG2, and T157, gave positive reactions on one or both of the phenolic substrates. One of these, a British isolate (BX1), gave phenolase reactions on both substrates.

Oxidation of Lignin

The fungi were screened for their ability to oxidize lignin by determining their reaction on wood-meal medium (Table III). All the cultures of the white-rot fungus, *P. tomentosus*, caused an oxidative browning of the wood meal but there was no evidence of oxidation with cultures of *C. puteana* and *C. sarcoides*. Since the fungi grew well on this medium and all the cultures produced changes in the pH, it is concluded that the essential enzyme which acts on lignin is lacking in the latter two species.

Decaying Activity

The ability of *C. sarcoides* to cause decay in spruce heartwood was first tested with isolate C52 in duplicate series on wood blocks which were adjusted

to the following moisture contents: 27, 35, 45, 60, 100, 125, 150, and 175% (oven-dry weight basis). No loss in weight occurred on any of the blocks during a period of 3 months. The remaining isolates of *C. sarcoides* together with the isolates of the wood-destroying fungi were tested in triplicate on wood blocks which rested on wood meal enriched with Badcock's accelerator. The three isolates of *C. puteana* caused an average loss in weight of 12%, whereas the average loss in weight for the four isolates of *P. tomentosus* was 3.3%. Very good agreement was obtained between replicate blocks and between the different isolates. In the tests with *C. sarcoides*, except for the two British isolates BA3 and BG2, which caused small losses of 0.6% and 1.0% respectively in only one block from each series, this fungus did not appear to be capable of causing decay.

Interaction of the Fungi

The interaction between *C. sarcoides* and the two decay fungi was tested on wood and on malt agar. For the tests on wood, 15 cultures each of isolate T157 and A29 of *C. sarcoides* were first grown on malt agar in screw-top jars for 5 to 6 weeks. Six additional jars were prepared in the same way but were not inoculated. Thirty-six test blocks were prepared with a cavity about $\frac{3}{4}$ in. in diameter drilled in one side of each block to take a disk of inoculum. After sterilization with steam, one block was placed in each of the culture jars with the fungi. Water was added to the blocks to give an initial moisture content of 50% of their dry weight, and the cultures were incubated at room temperature in a saturated atmosphere for 2 months. When infection was confirmed in the inoculated blocks by the presence on the wood of a pink or purple stain, or by fruiting bodies of the fungus, the blocks were removed aseptically from the agar cultures and placed in washed and sterilized jars without agar. Disks of wood which had been incubated with cultures of the wood-destroying fungi for 3 months were used to inoculate the test blocks. The procedure was then as follows:

Blocks infected with *C. sarcoides* (15 blocks of isolates T157 and A29)
- 3 inoculated with *C. puteana* (C5)
- 3 " " *P. tomentosus* (A20)
- 3 steamed and then inoculated with *C. puteana* (C5)
- 3 " " " " " *P. tomentosus* (A20)
- 3 blocks no further treatment

Blocks not infected with *C. sarcoides*
- 3 inoculated with *C. puteana* (C5)
- 3 inoculated with *P. tomentosus* (A20)

This experiment showed that isolate A29 of *C. sarcoides* was able to retard considerably decay by *C. puteana* and *P. tomentosus*. With *C. puteana*, the loss in weight, i.e. the rate of decay, was reduced by about 75% of that obtained in the controls where *C. sarcoides* was absent, or in the blocks where the fungus had been killed by steam. With *P. tomentosus*, the rate of decay was reduced by about one-half of that obtained in the controls, and almost two-thirds of that obtained in the blocks which had been steamed. Neither the living or dead mycelia of isolate T157 of *C. sarcoides* appeared to have any

measurable effect on the rate of decay of the wood-destroying fungi. There was no loss in weight after 5 months in the blocks inoculated with only the cultures of *C. sarcoides*.

To investigate the nature of the interaction between *C. sarcoides* and one of the decay fungi, 12 isolates of the former were screened for their antagonism against isolate C5 of *C. puteana*. Five British and seven Canadian isolates were tested as follows: an agar plug from an actively growing plate culture of *C. puteana* was placed at one edge of a plate containing 20 ml. of malt agar, and a spore suspension of *C. sarcoides* was streaked on the opposite side of the plate at a distance of about 6 cm. The width of the zone between the two cultures was measured at the end of 7, 10, and 14 days, and observations were made on the mutual effects of the growth of the two fungi. The results obtained at the end of 14 days are summarized in Table IV.

TABLE IV

ANTAGONISTIC ACTION OF *Coryne sarcoides* AGAINST *Coniophora puteana* (C5), AS DETERMINED BY WIDTH OF INHIBITION ZONE BETWEEN COLONIES OF THE FUNGI ON MALT AGAR AT THE END OF 14 DAYS

Isolate of *Coryne sarcoides*	Width of inhibition zone between the two colonies (cm.)	Remarks
BX1 (British)	5.0	Inhibition zone completely encircles *C. sarcoides*
BA3	—	*C. sarcoides* half overgrown by *C. puteana*
BG2	6.0	Aerial mycelium of *C. puteana* growing over zone in one place
A11	—	*C. sarcoides* completely overgrown by *C. puteana*
FC1	—	*C. sarcoides* two-thirds overgrown by *C. puteana*
L2C (Canadian)	—	*C. sarcoides* about half overgrown by *C. puteana*
C103	—	*C. sarcoides* almost completely overgrown by *C. puteana*
A29	3.0	Aerial mycelium of *C. puteana* growing over zone in one place
A110	—	*C. sarcoides* completely overgrown by *C. puteana*
C52	—	*C. sarcoides* two-thirds overgrown by *C. puteana*
T157	—	*C. sarcoides* completely overgrown by *C. puteana*
A56	—	*C. sarcoides* about one-third overgrown by *C. puteana*
Control: *Coniophora puteana* (C5), alone		Plate almost completely overgrown

Isolates BX1, BG2 (British), and A29 (Canadian) of *C. sarcoides* prevented the growth of *C. puteana* at distances of 5.0, 6.0, and 3.0 cm. respectively, but the remaining nine isolates of this fungus were overgrown to a greater or less extent by the decay fungus. The tests with isolates A29 and T157 of *C. sarcoides* were of some value in explaining the results obtained from the decay experiments, since isolate A29 which retarded decay by *C. puteana* in the wood blocks also prevented growth of this fungus on agar (Fig. 1). In contrast, culture T157 was inactive on agar, and did not affect the fungus on wood.

It is noteworthy that 40% of the British isolates of *C. sarcoides* were antagonistic in these tests, while only one, representing 14% of the Canadian isolates, was active.

Discussion

Some information on the role of *C. sarcoides* in the decay complex of living trees has been gained from these studies. On the basis of laboratory tests it can be stated with reasonable certainty that this fungus does not cause loss in weight of wood, although it produces enzymes, i.e., cellulases and polyphenol oxidases similar to those formed by wood-destroying fungi. The action of *C. sarcoides* on wood is as yet unknown, although the production of cellulase by wood-destroying fungi, particularly fungi of the brown-rot type, is obviously associated with their ability to decompose wood. It is possible that the cellulolytic action of *C. sarcoides* is too slow on wood to be measured by the loss in weight method, or that basic differences in metabolism are involved. However, little is known regarding the metabolism of wood-inhabiting fungi generally and to what extent it may differ in those fungi which show little or no ability to cause decay. For example, the relative loss in weight of substrate might not be a valid basis for comparing the decaying abilities of fungi if a larger part of the substrate is converted into cell material by some fungi than by others. The relatively high loss in weight caused by *C. puteana* in these tests suggests that a large amount of the substrate is converted to CO_2 during metabolism. This might not be the case with *C. sarcoides* where a larger amount of the substrate carbon may be accumulated in the mycelium of the fungus. A further comparison of these fungi to determine the fate of the substrate carbon may serve to elucidate this point.

The outstanding characteristic of *C. sarcoides* is the production by certain strains of an antibiotic which is capable of retarding the development of wood-destroying fungi. Although studies with this fungus show that many of the isolates were quite inactive, several were very active in preventing the growth of *C. puteana* on agar plates, and the antagonism of one isolate was shown to the extent that decay by *C. puteana* and *P. tomentosus* was reduced by from 50 to 75% of that obtained in the controls. However, the real significance of this characteristic of *C. sarcoides* depends on the relative occurrence of active antagonistic strains in nature. This has yet to be determined.

FIG. 1. Antagonistic action of *C. sarcoides* (upper colony) on *C. puteana* (C-5), 2 weeks after inoculation of the plates.
Culture No. T157. Inactive isolate of *C. sarcoides*.
Culture No. A-29. Active isolate of *C. sarcoides*.

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